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<b>(21) International Application Number:</b> PCT/US96/09613 <b>(22) International Filing Date:</b> 7 June 1996 (07.06.96)  <b>(30) Priority Data:</b> 08/474,673 7 June 1995 (07.06.95) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/474,673 (CIP) Filed on 7 June 1995 (07.06.95)  <b>(71) Applicant (for all designated States except US):</b> SMITHKLINE BEECHAM CORPORATION [US/US]; Corporate Intellectual Property, UW2220, 709 Swdeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> YOUNG, Peter, Ronald [US/US]; 32 Hendrickson Road, Lawrenceville, NJ 08648 (US). ERICKSON-MILLER, Connie, L. [US/US]; 608 Brainerd Place, Exton, PA 19341 (US).		<b>(74) Agents:</b> JERVIS, Herbert, H. et al.; SmithKline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swdeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).  <b>(81) Designated States:</b> AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title: METHOD FOR OBTAINING RECEPTOR AGONIST ANTIBODIES</b>		
<b>(57) Abstract</b>		
<p>A method is provided for generating antibodies which demonstrate the agonist properties of the naturally occurring ligand of a receptor molecule by using as an immunogen a recombinant immunogen which corresponds to a multimeric form of a receptor. In this immunogen the extracellular domains are in a similar disposition to that expected for receptors having two or more subunits on the surface of the cell.</p>		

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## METHOD FOR OBTAINING RECEPTOR AGONIST ANTIBODIES

Cross Reference to Related Applications

- 5           This application is a continuation-in-part of co-pending U.S. application serial number 08/474,673, filed June 7, 1995, the contents of which is incorporated hereby by reference.

Field of the Invention

- 10           The present invention relates generally to the generation of monoclonal antibodies by use of a selected antigen; and more particularly to the generation of monoclonal antibodies which are receptor agonists.

Background of the Invention

- 15           A vast majority of receptors of the single transmembrane class respond to ligand binding by some form of aggregation. This aggregation can be between identical receptor subunits (as in homodimerization, homotrimerization, etc.) or between different receptor subunits (as is heterodimerization, heterotrimerization, etc.). This aggregation appears to be part of the signal for the target cell to respond  
20           biologically, in that mutants of the ligand which are unable to interact with the second subunit are still able to bind, but no longer cause dimerization and biological activation of the receptor [P. R. Young, Curr. Opin. Biotech., 3:408-421 (1992)].

- For example, there is evidence in the literature that suggests dimerization of the erythropoietin receptor (EpoR) upon ligand binding [S. S. Watowich *et al.*,  
25           Molec. Cell Biol., 14:3535-3549 (1992) and S. S. Watowich *et al.*, Proc. Natl. Acad. Sci. USA, 89:2140-2144 (1992)]. Reports about IL-6 have indicated that its second subunit gp130 may dimerize upon IL-6 binding [M. Murkami *et al.*, Science, 260:1808-1810 (1993)]. For some receptors in which homodimerization is induced by ligand binding, monoclonal antibodies (mAbs) were discovered which had  
30           agonist properties. These include mAbs to EGF, TNF and growth hormone receptors [A. B. Schrieber *et al.*, J. Biol. Chem., 258:846-853 (1983); L. H. K.

Defize *et al.*, EMBO J., 5:1187-1192 (1986); H. Engelmann *et al.*, J. Biol. Chem., 265:14497-14504 (1990); and G. Fuh *et al.*, Science, 256:1677-1680 (1992)]. In each case, these mAbs, by virtue of their two antigen recognition sites, were able to bring together two receptors and thus activate them. Fab fragments made from these mAbs were inactive. In some cases, the apparent affinity of the antibody for receptor was comparable to that of the ligand, e.g., growth hormone [Fuh *et al.*, cited above].

It has also been discovered that antibodies to IL-3 receptor have agonist properties [Suguwara *et al.*, J. Immunol., 140:526-530 (1988)]. Previous literature has described the production of anti-erythropoietin receptor antibodies [A. D'Andrea *et al.*, Blood, 82:46-52 (1993); A. D'Andrea *et al.*, Blood, 84:1982-1991 (1994) and M-G Yet *et al.*, Blood, 82: 1713-1719 (1993). See also, PCT Application WO96/03438 published 8 February 1996. While the Yet *et al.*, reference suggests the occurrence of possible EPO-like activity in one mAb, the mAb is not characterized. Neither Yet *et al.*, nor the other literature provides any reproducible manner of generating agonist mAbs.

There remains a need in the art for the development of additional mAbs which have an affinity for receptors comparable to that of the ligand, and which can act as agonists of the receptor.

## Summary of the Invention

In one aspect, the present invention provides a method for reliably generating an antibody which is an agonist of a receptor. This method employs as the immunizing antigen, a recombinant immunogen which consists of a first extracellular domain of a receptor molecule spaced apart from a second extracellular domain from that receptor by a bridging moiety. The bridging moiety places the first domain and the second domain into a functional proximity which mimics the functional domain orientation and proximity of the naturally occurring multimeric receptor. The bridging moiety can be an amino acid spacer peptide, an organic molecule, an Fc portion of a human immunoglobulin or an amphipathic helix, for example.

In another aspect, the invention includes antibodies produced by the above-described method. The antibodies so generated are characterized by the ability to bind to the naturally occurring receptor and by such binding initiate the biological activity of the receptor. The antibodies of the invention may be chimeric antibodies,  
5 humanized antibodies, monoclonal antibodies or polyclonal antibodies.

In still another aspect, the invention provides a recombinant polynucleotide sequence comprising a nucleotide sequence encoding the extracellular domain of a receptor molecule fused in frame to a nucleotide sequence encoding a specific proteolytic cleavage site, said cleavage sequence associated with a bridging moiety.

10 Yet a further aspect of the invention is the recombinant multimeric immunogen itself.

Additional aspects of this invention include a vector comprising a polynucleotide sequence described above under the control of suitable regulatory sequences capable of directing replication and expression of the polynucleotide  
15 sequence in a host cell, and a transformed host cell.

Still other aspects of the invention include therapeutic reagents comprising the antibodies produced by the method of this invention, as well as a method of treating a disease condition by administering a pharmaceutical composition of the invention.

20 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

#### Brief Description of the Drawings

Fig. 1 is a graph illustrating the effects of the dimeric fusion protein EpoRFc and the monomeric protein mEpoR on erythropoietin stimulation of UT-7 Epo cells,  
25 which plots % positive control vs. concentration (ng/ml).

Fig. 2 is a graph illustrating the effects of the dimeric fusion protein EpoRFc and the monomeric protein mEpoR on erythropoietin stimulation of DA3Epowt cells, which plots % positive control vs. concentration (ng/ml).

Fig. 3 is a graph illustrating the effects of the dimeric fusion protein EpoRFc and the monomeric protein mEpoR on erythropoietin stimulation in an CFU-E assay, which plots % positive control vs. concentration (ng/ml).

Fig. 4 is a bar graph demonstrating the binding of antibodies developed in response to immunization with the EpoRFc fusion protein to 32D/Epo wt and parental 32D Fig. (4a) and UT7-EPO, cells, plotting %Isotype control log fluorescence vs. control (CTL) and supernatant designations.

Fig. 5 is a bar graph demonstrating the results of an UT7-Epo proliferation assay with three mAbs to EpoRFc, plotting %Epo maximum vs. dilutions of supernatants. The supernatant designations are listed above the bars.

Fig. 6 is a bar graph demonstrating the ability of 4 mAbs to EPORF<sub>c</sub> to stimulate the proliferation and differentiation of human bone marrow progenitor cells to form red blood cell colonies (CFU-E), plotting Numbers of CFU-E vs. positive and negative EPO controls and supernatant dilutions. The supernatant designations are listed above the bars.

Fig. 7 is the DNA sequence [SEQ ID NO: 1] for plasmid mtalsEpoRFc containing the sequence encoding the EpoRFc fusion protein under control of a *Drosophila* S2 mtn promoter. Nucleotides 1 to 897 contain the promoter sequence. The EpoRFc fusion protein [SEQ ID NO: 2] is encoded by the following: nucleotides 898 to 1647 encode the EpoR extracellular domain protein; nucleotides 1648 to 1659 encode the Factor Xa cleavage sequence; nucleotides 1660 to 2361 encoding the human IgG<sub>1</sub> Fc sequence. The remainder of the sequence is derived from the plasmid parent. See Example 1.

Fig. 8 is the DNA sequence [SEQ ID NO: 3] of CosFcLink vector from which a KpnI/XbaI insert containing the IgG1 Fc region was obtained.

#### Detailed Description of the Invention

The present invention provides a reproducible and reliable composition and method for generating antibodies which demonstrate the agonist properties of the naturally occurring ligand of a receptor molecule. While, in principle, any purified preparation could be used to generate antibodies to a receptor, and from these some

may be agonists, this invention provides a specific immunogen which corresponds to a multimeric form of a receptor in which the extracellular domains are in a similar disposition to that expected for receptors having two or more subunits on the surface of the cell.

5

### *I. The Recombinant Immunogen*

The recombinant immunogen of the present invention consists of an extracellular domain of a selected receptor molecule which is involved in ligand binding via the interaction of more than one extracellular domain. The extracellular domain is spaced apart from a second extracellular receptor domain by a bridging moiety.

By "extracellular domain" is meant that portion of a receptor protein which is localized on the extracellular surface of a cell and which contributes to interaction and binding with its native ligand.

The receptor subunit which contributes the extracellular domain(s) of the immunogen may be any receptor subunit which accomplishes ligand binding via a homodimer of the extracellular domain, a heterodimer of two different subunits of the receptor's extracellular domain, or a multimer of subunits. Receptors which homodimerize upon ligand binding and thus may contribute extracellular domains to the recombinant immunogen include those for, *inter alia*, erythropoietin (EPO) and thrombopoietin (CMPL), G-CSF, M-CSF, TGF- $\alpha$ , EGF, neu, growth hormone, prolactin, placental lactogen, c-kit (stem cell factor receptor), p50 and p75 receptor subunits of TNF $\alpha$ , and TNF $\beta$ . Other receptors which are anticipated to dimerize based on homology to TNF and which can be included in this list are Fas, CD40, CD27, CD30, 4-1BB and OX40.

Similarly, receptors which are already in a homodimeric form on the cell surface, prior to ligand binding may also contribute the extracellular domain(s) to the immunogen. These latter receptors include, *inter alia*, insulin, IGF1 and IGF2, and PDGF. PDGF includes dimeric ligand made of two chains A and B and two receptor subunits a and b. Ligands and receptors can associate as homo- or hetero-

dimers. Relaxin is also anticipated to be a dimeric receptor prior to ligand binding based on its homology to insulin and IGF.

Receptors which are formed by heterodimers of two different subunits may also be employed as contributors of extracellular domain(s) of one or both of the subunits to the recombinant. Such receptors include, *inter alia*, GM-CSF, IL-3, IL-5, IL-6, Oncostatin M, CNTF, LIF, NGF, FGF, IL-4, IL-13, IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$ , TGF $\beta$ 1, TGF $\beta$ 2 and IL-12. Receptors, such as IL-3, which involve more than one subunit may respond to this method, if dimerization of one of the subunits is required for signal transduction.

Receptors which form other aggregations, such as trimers of IL-2 receptor subunits, may also be used as sources of extracellular domain(s) for the recombinant immunogen of this invention.

The extracellular domains of the selected receptor useful in the recombinant immunogen may be isolated and/or otherwise obtained by resort to the published and publicly available receptor sequences. Methods conventional in the art may be employed to isolate or synthesize the appropriate nucleotide sequences encoding these domains for further manipulation in the generation of the immunogen of this invention.

By the term "bridging moiety" is meant a peptide or non-peptide sequence that stably associates with itself and places the first extracellular domain and the second extracellular domain into functional proximity (i.e., a relationship between the two or more domains which mimics the three dimensional functional proximity of the domains in the naturally occurring multimeric receptor, during or prior to ligand binding. Preferably, the bridging moiety is of a sufficient size to bring together the membrane proximal regions (i.e., the regions closest to the transmembrane region) of the extracellular domain. The precise dimensions of the bridging region can be estimated from the relevant crystal structures, such as those for growth hormone and its receptor, or TNF and its receptor [see, e.g., DeVos *et al.*, Science, 255:306 (1992)].

The suitability of a given "bridging moiety" can sometimes be evaluated empirically. For example, a dimeric form of a receptor which dimerizes upon



binding of ligand is expected to have a higher affinity for ligand than a monomeric form of the extracellular domain of the receptor, if the bridging group is appropriate.

This would be evidenced either by direct binding studies of ligand to monomeric and dimeric receptor, or by the relative ability of the two forms of receptor to  
5 neutralize the biological activity of the ligand. For example, Fig. 1 shows the ability of dimeric EpoRFc to neutralize Epo activity about 100 times more effectively than monomeric EpoR.

An example of a bridging moiety is an amino acid spacer sequence (e.g., between 1 to 10 amino acids in length and optionally encoding a cleavage site) fused  
10 to a dimerization or oligomerization domain which permits the formation of dimers or oligomers, respectively. In the following example, for instance, a spacer encoding the Factor Xa cleavage site is part of the bridging moiety; and the dimerization domain is the hinge CH<sub>2</sub>CH<sub>3</sub> region of a human IgG1. This IgG component consists of the CH<sub>2</sub> and CH<sub>3</sub> domains and the hinge region of IgG1  
15 including cysteine residues contributing to inter-heavy chain disulfide bonding, for example residues 11 and 14 of the IgG1 hinge region [see, e.g., B. Frangione, Nature, 216: 939-941, (1967)]. Preferably the IgG1 component consists of amino acids corresponding to residues 1-4 and 6-15 of the hinge, 1-110 of CH<sub>2</sub> and 1-107 of CH<sub>3</sub> of IgG1 described by J. Ellison *et al.*, Nucleic Acids Res., 10: 4071-4079  
20 (1982). Residue 5 of the hinge is changed from cysteine in the published IgG1 sequence to alanine by alteration of TGT to GCC in the nucleotide sequence.

The bridging moiety can also be an Fc portion of any human immunoglobulin with an intact hinge CH<sub>2</sub>CH<sub>3</sub> region, including those derived from IgG, IgE, IgM, IgA and IgG<sub>4</sub>. Still another suitable bridging moiety is an amino  
25 acid spacer fused to the C terminal domain of the TNF-like receptor of the Shope fibroma poxvirus [C. A. Smith *et al.*, BBRC, 176: 335-342 (1991)]. The bridging moiety may also be an a dimerization domain like an amphipathic helix, such as a leucine zipper [see, e.g., P. Pack *et al.*, Biochem., 31(6): 1579-1584 (Feb. 18, 1992)]. The bridging moiety may also be alkaline phosphatase.

30 Still other desirable embodiments of the bridging moiety are organic molecules which can functionally associate the receptor extracellular domains as

desired. Such organic molecules may be selected from among such molecules known to associate peptide sequences to each other for other biological uses, e.g., bifunctional cross-linkers, such as carbodiimide, glutaraldehyde and DSS, BS3, and others which may be obtained from several commercial sources. However, these associations may require combination with specific target sequences for cross-linking, e.g., an exposed Cys or His for nickel chelate, to achieve the appropriate three dimensional disposition of receptor subunits. Choice of an appropriate cross-linker can be determined by comparison to known crystal structures of homologous receptors.

The suitability of a particular peptide or non-peptide entity as a bridging moiety may be functionally assessed in a receptor ligand binding assay. The suitability of the bridging moiety may be determined if the recombinant immunogen binds the receptor's intended ligand with greater affinity than does the monomeric sequence of the receptor extracellular domain. Ligand binding assays for the selected receptors are known to those of skill in the art and may be readily selected without undue experimentation. See, for example, the EpoR ligand assays described in Komatsu *et al.*, Blood, 82:456-464 (1993); Miura *et al.*, Mol. Cell. Biol., 11:4895-4902 (1991); Witthuhn *et al.*, Cell, 74:227-236 (1993).

Another bridging moiety or linker may be prepared by mutagenesis of a receptor in the membrane proximal domain to create unpaired Cys, which can disulfide bond to create a dimeric receptor. Such mutations can be evaluated for appropriateness by observing whether the full length receptor containing such a mutation is able to constitutively activate the ligand's activity upon transfection into suitable target cells (e.g., EpoR mutations) [see, e.g., Watowich *et al.*, Proc. Natl. Acad. Sci. USA., 89:2140-2144 (1992)].

Thus, for example, the bridging moiety of the recombinant immunogen can associate two identical extracellular domains into an immunogen that mimics a homodimeric receptor. Alternately, for example, the bridging moiety can associate two different extracellular domains of subunits of one receptor into an immunogen that mimics a heterodimeric receptor. For example, for the heterodimeric association of different subunits from a heterodimeric receptor, a different bridging

moiety could be used for each receptor subunit. The bridging moiety for a heterodimeric receptor is preferably a domain that cannot associate with itself, but which preferentially associates with a second domain. Thus, the first bridging moiety can be a CH1 region of a light chain of a selected immunoglobulin. Its  
5 complementary bridging moiety is the CH1 region of the heavy chain of the same immunoglobulin or the entire Fc region including the CH1, hinge, CH2, and CH3 regions of the heavy chain. It is also anticipated that various bridging entities may be employed in preparing other multimeric immunogens, e.g., trimers, by associating three identical domains or three extracellular domains from one, two or  
10 three subunits of a single receptor. It is presently preferred to use an Fc portion of an immunoglobulin as a bridging moiety to associate the extracellular receptor domains, as disclosed in Example 1 below.

## II. *Construction and Preparation of the Recombinant Immunogen*

15 The present invention also provides the nucleic acid sequences encoding the recombinant immunogens described above. The nucleotide sequences encoding the extracellular domains of the receptors useful in the immunogens may be obtained from known receptor sequences by conventional means [see, J. Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory  
20 (1989)]. For example, the nucleotide sequences which form the immunogen of the invention may be isolated by conventional uses of polymerase chain reaction or conventional genetic engineering cloning techniques. Alternatively, these sequences may be constructed using chemical synthesis techniques.

Optionally, a nucleotide sequence which encodes a peptide sequence which  
25 provides an enzymatic cleavage site (of which many are well known in the art) is fused in frame to the extracellular domain nucleotide sequence prior to its association with the dimerization or oligomerization domain of the bridging moiety. This facilitates cleavage of the extracellular domain from the bridging moiety following expression.

30 According to the invention, the nucleic acid sequences encoding the extracellular domains may be modified as desired. It is within the skill of the art to

obtain other polynucleotide sequences encoding these receptor domains useful in the invention. Such modifications at the nucleic acid level include, for example, modifications to the nucleotide sequences which are silent or which change the amino acids, e.g. to improve expression or secretion. Such modifications include  
5 amino terminal, carboxy terminal or internal deletions of the nucleotide sequences, as desired.

Where the bridging moiety is an amino acid sequence, the nucleotide sequence encoding it is also obtained conventionally and fused to the extracellular domain sequence directly or via a cleavage site or via additional sequence intended  
10 as a spacer. For example, where the bridging moiety is an Fc portion of a human immunoglobulin with intact hinge CH2CH3 region, the nucleotide sequence encoding the Fc region is obtained from known antibody sequences, prepared by conventional techniques and fused in frame to the receptor sequences or to the sequence providing the enzymatic cleavage site.

15 To produce recombinant immunogens of this invention, a DNA sequence of the invention encoding the extracellular receptor domain, is fused in frame to an optional cleavage site and further fused to a nucleotide sequence encoding a peptide bridging moiety. Preparation of the nucleic acid sequences may be carried out chemically, enzymatically, or by a combination of the two methods, in vitro or in  
20 vivo as appropriate. Thus, the DNA sequences may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts *et al.*, Biochem., 24:5090-5098 (1985). The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by  
25 enzymatic polymerization on DNA or RNA templates, or by a combination of these methods. These methods are generally provided by the commercial supplier of the reagents.

For example, digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20-70°C, generally in a volume of 50ul or less  
30 with 0.1-10ug DNA. Enzymatic polymerization of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an

appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10-37°C, generally in a volume of 50ul or less.

Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to ambient,

5 generally in a volume of 50ul or less. The chemical synthesis of the DNA sequence or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in "Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual" (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other  
10 scientific publications, for example M.J. Gait *et al.*, Nucleic Acids Res., 10: 6243 (1982) and others. Preferably an automated DNA synthesizer is employed. The DNA sequence is preferably prepared by ligating two or more DNA molecules which together comprise a DNA sequence encoding the compound. The DNA molecules may be obtained by the digestion with suitable restriction enzymes of  
15 vectors carrying the required coding sequences or by use of polymerase chain reaction technology. The precise structure of the DNA molecules and the way in which they are obtained depends upon the structure of the desired product. The design of a suitable strategy for the construction of the DNA molecule coding for the recombinant immunogen is a routine matter for one skilled in the art.

20 Once the nucleotide sequence encoding the recombinant immunogen is designed, it is inserted into a suitable expression system. Systems for cloning and expression of a selected protein in a desired microorganism or cell, including, e.g. *E. coli*, *Bacillus*, *Streptomyces*, mammalian, insect, and yeast cells, are known and available from private and public laboratories and depositories and from commercial  
25 vendors.

Desirably, a recombinant molecule or vector is constructed in which the polynucleotide sequence encoding the immunogen is operably linked to a heterologous expression control sequence permitting expression of the human protein. Numerous types of appropriate expression vectors are known in the art for  
30 eukaryotic (including human) protein expression, by standard molecular biology techniques. Such vectors may be selected from among conventional vector types

including mammalian, insects, e.g., baculovirus expression, *Drosophila* S2 cell, or yeast, fungal, bacterial or viral expression systems. Other appropriate expression vectors, of which numerous types are known in the art, can also be used for this purpose. Methods for obtaining such expression vectors are well-known. See, for example, Sambrook *et al.*, cited above; Miller *et al.*, Genetic Engineering, 8:277-298 (Plenum Press 1986) and Johansen *et al.*, Genes and Develop., 3:882-889 (1989)].

Suitable host cells or cell lines for transfection by this method include mammalian cells, such as Human 293 cells, Chinese hamster ovary cells (CHO), the monkey COS-1 cell line or murine 3T3 cells derived from Swiss, Balb-c or NIH mice may be used. Another suitable mammalian cell line is the CV-1 cell line. Still other suitable mammalian host cells, as well as methods for transfection, culture, amplification, screening, and product production and purification are known in the art. [See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman *et al.*, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley *et al.*, U. S. Patent 4,419,446].

Similarly bacterial cells are useful as host cells for the present invention. For example, the various strains of *E. coli* (e.g., HB101, MC1061, and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas*, *Streptomyces*, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Other fungal cells may also be employed as expression systems. Alternatively, insect cells such as *Spodoptera frugiperda* (Sf9) or *Drosophila* S2 may be used.

Thus, the present invention provides a method for producing a recombinant novel multimeric receptor immunogen which involves transfecting a host cell with at least one expression vector containing a recombinant polynucleotide as above-described under the control of a transcriptional regulatory sequence, e.g. by conventional means such as electroporation. The transfected host cell is then cultured under suitable conditions that allow expression of the product of the recombinant polynucleotide. During expression, the recombinant multimeric

immunogen is formed in the cell by the association of the bridging moiety with itself. The expressed multimeric protein is then recovered, isolated, and optionally purified from the culture medium (or from the cell, if expressed intracellularly) by appropriate means known to one of skill in the art.

- 5           To generate a heterodimeric receptor immunogen, a vector is designed as above to carry a first receptor subunit which is fused to, e.g., the CH1 region of the light chain. A vector is also designed to carry the second, different subunit of the receptor fused to e.g., the heavy chain sequence (e.g., CH1 region or the entire Fc region of the same Ig that contributes the light chain CH1, as described above).
- 10          Alternatively both sequences could be present on a single vector. The vectors are prepared as described above. However, for a heterodimeric receptor immunogen, the host cell must be co-transfected with both receptor-bridging moiety polynucleotide sequences. Expression of both sequences in the host cell causes the light chain CH1 and heavy chain CH1, or light chain CH1 and heavy chain Fc
- 15          sequences to associate only complementarily (i.e., with each other not with their identical counterparts in the cell). Heterodimeric receptor immunogens are thereby formed in the manner of a "Fab"-like fragment or an antibody-like fragment, respectively.

- Once expressed, the recombinant immunogen (e.g., homodimeric or
- 20          heterodimeric) may be isolated following cell lysis in soluble form, or may be extracted using known techniques, e.g., in guanidine chloride. If the protein is secreted, it can be isolated from culture supernatant and purified. With an Fc bridging moiety, Protein A or Protein G Sepharose may be used to purify the immunogen. Where the immunogen is a receptor subunit sequence fused to a
- 25          peptide epitope, a specific monoclonal antibody mAb to the peptide epitope can be used to purify the immunogen.

- Another method of producing the novel multimeric receptor immunogen involves directly injecting the monomeric recombinant DNA (as, e.g., "naked DNA") into mice or rabbits intramuscularly. The multimeric receptor thus
- 30          assembles *in vivo*, where it acts as an immunogen. See, e.g., Cohen, Science,

259:1691-92 (1993); E. F. Fynan *et al.*, Proc. Natl. Acad. Sci., USA, 90:11478-11482 (1993).

Association of a nucleotide sequence encoding the selected receptor extracellular domain and its optional cleavage site with a non-peptide bridging moiety may be by conventional covalent or ionic bonds, using conventional chemical linking agents. If the association is non-covalent, then cross-linking must occur either after purification or in freshly isolated cells prior to purification. Alternatively, the opportunity to cross-link could be enhanced by adding ligand to the receptor sequences to bring them into proximity to each other, add cross-linking agent and bridging moiety, and dissociate ligand by a conventional technique, e.g., low pH. The dimeric receptor could then be purified.

### III. *The Method of the Invention*

The recombinant immunogens of this invention are thus useful as antigens for the development of anti-receptor antisera and antibodies to the multimeric receptor domain immunogen. Specific antisera and polyclonal antibodies may be generated by employing the recombinant multimeric immunogen as an immunogen using known techniques. See, Sambrook, cited above, Chapter 18, generally, incorporated by reference. Additionally, polyclonal antibodies and antisera may be generated to the immunogen formed *in vivo* following administration of the naked DNA.

The polyclonal antibodies developed in the immunized animal may be isolated from the animal's plasma, peripheral blood or other tissue in a conventional manner. Antibodies thus isolated may be employed in the methods described below for generation of mAbs, humanized and chimeric antibodies of the invention.

For example, monoclonal antibodies of the invention may be produced by conventional methods, including the Kohler and Milstein hybridoma technique, in which spleen cells from an immunized animal are fused with immortalized cells to create hybridoma cell lines which secrete a single mAb. Each hybridoma is then screen with a simple binding assay to detect agonist properties.



Other types of antibodies may be designed based on the agonist mAbs so identified. For example, recombinant techniques, such as described by Huse *et al.*, Science, 246:1275-1281 (1988), or any other modifications thereof known to the art may be employed to generate antibodies. Thus, also encompassed within this  
5 invention are methods for generating humanized and chimeric agonist antibodies by employing the CDRs from the agonist antibodies produced as described above. Methods of identifying suitable human framework regions and modifying a mAb of the invention to contain same to produce a humanized or chimeric antibody of the invention, are well known to those of skill in the art. See, e.g., E. Mark and Padlin,  
10 "Humanization of Monoclonal Antibodies", Chapter 4, The Handbook of Experimental Pharmacology, Vol. 113, The Pharmacology of Monoclonal Antibodies, Springer-Verlag (June, 1994). Other types of recombinantly-designed antibodies are also encompassed by this invention.

As used in this specification and the claims, the following terms are defined  
15 as follows:

"Altered antibody" refers to a protein encoded by an altered immunoglobulin coding region, which may be obtained by expression in a selected host cell. Such altered antibodies are engineered antibodies (e.g., chimeric, humanized, reshaped human or reconstituted human antibodies) or antibody fragments lacking all or part  
20 of an immunoglobulin constant region, e.g., Fv, Fab, or F(ab')<sub>2</sub> and the like.

"Altered immunoglobulin coding region" refers to a nucleic acid sequence encoding the altered antibody of the invention. When the altered antibody is a "reshaped human antibody", the sequences that encode the complementarity determining regions (CDRs) from a donor human immunoglobulin are individually  
25 inserted into a first immunoglobulin partner comprising human variable framework or as components of a variable region gene sequence attached to human constant sequences. If desired, the first immunoglobulin partner is operatively linked to a second fusion partner.

"First immunoglobulin partner" refers to a nucleic acid sequence encoding a  
30 human framework or human immunoglobulin variable region in which the native (or naturally-occurring) CDR-encoding regions are replaced by the CDR-encoding

regions of a donor human antibody. The human variable region can be an immunoglobulin heavy chain, a light chain (or both chains), an analog or functional fragments thereof. Such CDR regions, located within the variable region of antibodies (immunoglobulins) can be determined by known methods in the art. For example Kabat *et al.* (Sequences of Proteins of Immunological Interest, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health (1987)) disclose rules for locating CDRs. In addition, computer programs are known which are useful for identifying CDR regions/structures.

"Second fusion partner" refers to another nucleotide sequence encoding a protein or peptide to which the first immunoglobulin partner is fused in frame or by means of an optional conventional linker sequence (i.e., operatively linked). Preferably the fusion partner is an immunoglobulin gene and when so, it is referred to as a "second immunoglobulin partner". The second immunoglobulin partner may include a nucleic acid sequence encoding the entire constant region for the same (i.e., homologous - the first and second altered antibodies are derived from the same source) or an additional (i.e., heterologous) antibody of interest. It may be an immunoglobulin heavy chain or light chain (or both chains as part of a single polypeptide). The second immunoglobulin partner is not limited to a particular immunoglobulin class or isotype. In addition, the second immunoglobulin partner may comprise part of an immunoglobulin constant region, such as found in a Fab, or F(ab)<sub>2</sub> (i.e., a discrete part of an appropriate human constant region or framework region). A second fusion partner may also comprise a sequence encoding an integral membrane protein exposed on the outer surface of a host cell, e.g., as part of a phage display library, or a sequence encoding a protein for analytical or diagnostic detection, e.g., horseradish peroxidase,  $\beta$ -galactosidase, etc.

The terms Fv, Fc, Fd, Fab, or F(ab')<sub>2</sub> are used with their standard meanings (see, e.g., Harlow *et al.*, Antibodies A Laboratory Manual, Cold Spring Harbor Laboratory, (1988)).

As used herein, an "engineered antibody" describes a type of altered antibody, i.e., a full-length synthetic antibody (e.g., a chimeric, reconstituted human, or reshaped human antibody as opposed to an antibody fragment) in which a portion

of the light and/or heavy chain variable domains of a selected acceptor antibody are replaced by analogous parts from one or more donor antibodies which have specificity for the selected epitope. For example, such molecules may include antibodies characterized by an engineered heavy chain associated with an unmodified light chain (or chimeric light chain), or vice versa. Engineered antibodies may also be characterized by alteration of the nucleic acid sequences encoding the acceptor antibody light and/or heavy variable domain framework regions in order to retain donor antibody binding specificity. These antibodies can comprise replacement of one or more CDRs (preferably all) from the acceptor antibody with CDRs from a donor antibody described herein.

A "chimeric antibody" refers to a type of engineered antibody which contains naturally-occurring variable region (light chain and heavy chains) derived from a donor antibody in association with light and heavy chain constant regions derived from an acceptor antibody from a heterologous species.

A "humanized antibody" refers to a type of engineered antibody having its CDRs derived from a non-human donor immunoglobulin, the remaining immunoglobulin-derived parts of the molecule being derived from one (or more) human immunoglobulin(s). In addition, framework support residues may be altered to preserve binding affinity (see, e.g., Queen *et al.*, Proc. Nat'l. Acad. Sci. USA, 86:10029-10032 (1989), Hodgson *et al.*, Bio/Technology, 9:421 (1991)).

The term "reconstituted human antibody" refers to an antibody in which a Fab is converted into a full length Mab by cloning the heavy chain of the Fab into a human Ig constant region comprising the hinge region and CH-2 and CH-3 domains. Preferably the constant region is one of the IgG isotypes IgG1- IgG4 or variants thereof such as PE mutations. A reconstituted human antibody also includes variants of the processes, mature NH<sub>2</sub> terminal regions of the light or heavy chain are altered to conform with the predicted germ line parent sequence.

The term "donor antibody" refers to an antibody (monoclonal, or recombinant) which contributes the nucleic acid sequences of its variable regions, CDRs, or other functional fragments or analogs thereof to a first immunoglobulin partner, so as to provide the altered immunoglobulin coding region and resulting

expressed altered antibody with the antigenic specificity and neutralizing activity characteristic of the donor antibody. One donor antibody suitable for use in this invention is 1C8.

5       The term "acceptor antibody" refers to an antibody (monoclonal, or recombinant) from a source genetically unrelated to the donor antibody, which contributes all (or any portion, but preferably all) of the nucleic acid sequences encoding its heavy and/or light chain framework regions and/or its heavy and/or light chain constant regions to the first immunoglobulin partner. Preferably a human antibody is the acceptor antibody.

10       "CDRs" are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. See, e.g., Kabat *et al.*, Sequences of Proteins of Immunological Interest, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health (1987). There are three heavy chain and three light  
15 chain CDRs (or CDR regions) in the variable portion of an immunoglobulin. Thus, "CDRs" as used herein refers to all three heavy chain CDRs, or all three light chain CDRs (or both all heavy and all light chain CDRs, if appropriate). CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest in this invention are derived from donor antibody variable  
20 heavy and light chain sequences, and include analogs of the naturally occurring CDRs, which analogs also share or retain the same antigen binding specificity and/or neutralizing ability as the donor antibody from which they were derived.

By "sharing the antigen binding specificity or neutralizing ability" is meant, for example, that although antibody such as 1C8 may be characterized by a certain  
25 level of antigen affinity, a CDR encoded by a nucleic acid sequence of 1C8 in an appropriate structural environment may have a lower, or higher affinity. It is expected that CDRs of 1C8 in such environments will nevertheless recognize the same epitope(s) as in 1C8. A "functional fragment" is a partial heavy or light chain variable sequence (e.g., minor deletions at the amino or carboxy terminus of the  
30 immunoglobulin variable region) which retains the same antigen binding specificity and/or neutralizing ability as the antibody from which the fragment was derived.

An "analog" is an amino acid sequence modified by at least one amino acid, wherein said modification can be chemical or a substitution or a rearrangement of a few amino acids (i.e., no more than 10), which modification permits the amino acid sequence to retain the biological characteristics, e.g., antigen specificity and high  
5 affinity, of the unmodified sequence. For example, (silent) mutations can be constructed, via substitutions, when certain endonuclease restriction sites are created within or surrounding CDR-encoding regions.

Analogues may also arise as allelic variations. An "allelic variation or  
10 modification" is an alteration in the nucleic acid sequence encoding the amino acid or peptide sequences of the invention. Such variations or modifications may be due to degeneracy in the genetic code or may be deliberately engineered to provide desired characteristics. These variations or modifications may or may not result in alterations in any encoded amino acid sequence.

The term "effector agents" refers to non-protein carrier molecules to which  
15 the altered antibodies, and/or natural or synthetic light or heavy chains of the donor antibody or other fragments of the donor antibody may be associated by conventional means. Such non-protein carriers can include conventional carriers used in the diagnostic field, e.g., polystyrene or other plastic beads, polysaccharides, e.g., as used in the BIAcore (Pharmacia) system, or other non-protein substances  
20 useful in the medical field and safe for administration to humans and animals. Other effector agents may include a macrocycle, for chelating a heavy metal atom, or radioisotopes. Such effector agents may also be useful to increase the half-life of the altered antibodies, e.g., polyethylene glycol.

Also provided by the invention are human antibodies derived from human  
25 hybridomas, antibodies obtained by rescue from transgenic mice expressing human Ig domains, and antibodies made in primates. Any other modifications which are known to be useful to prepare mAbs as pharmaceutical agents may also be made to the antibodies of this invention.

Without wishing to be bound by theory, it is anticipated that the recombinant  
30 immunogens of this invention have configurations mimicking the form of the multimeric receptor on the cell surface. Thus, these immunogens generate a higher

frequency of antibodies which crosslink the multiple receptor subunits in a manner similar to that of the naturally occurring ligand and thereby are more likely than randomly obtained antibodies to be agonists of the receptor.

5    **IV.    Utilities**

Polyclonal antisera, monoclonal antibodies and other antibodies of this invention, which bind to the novel immunogen as the antigen and can function as agonists are useful in substantially the same manner as is the naturally occurring ligand of the receptor. For example, an agonist antibody developed to the exemplary  
10 erythropoietin receptor dimeric fusion protein described in Example 2 may be used in therapeutic, diagnostic and research methods in which the ligand, erythropoietin, is useful. These antibodies may be used as research tools and as components for separation of the receptor proteins from other contaminants of living tissue, for example, are also contemplated for these antibodies.

15       Agonist antibodies to the receptor would have the same therapeutic utility as the natural ligand, but would have the advantage of longer half-life and hence prolonged activity *in vivo*. These agonists can thus be employed to activate the biological activity which results from receptor/ligand binding. Thus, these agonist antibodies are useful in the treatment of diseases in which the interaction of the  
20 receptor and its ligand is part of a biochemical cascade of events leading to a desired response. The advantages of such agonist antibodies include the ability to administer lower dosages of antibody than ligand, easier and less frequent administration of a pharmaceutical based on the agonist antibody, as well as easier purification. Agonist antibodies may demonstrate a different profile of activity *in*  
25 *vivo* than the non-agonist antibodies due to a different distribution.

Compositions and methods useful for the treatment of conditions associated with abnormal receptor or ligand levels are provided. The present invention provides pharmaceutical compositions useful in the treatment of :anemia associated with chronic renal failure; anemia associated with AIDS; pre-dialysis patients;  
30 patients in need of pre- and/or post surgery hematocrit boosting; cancer patients undergoing hematocrit decreasing radiation or chemotherapy; rheumatoid arthritis

and sickle cell anemia. These compositions contain a therapeutically effective amount of an agonist antibody of this invention and an acceptable pharmaceutical carrier. As used herein, the term "pharmaceutical" includes veterinary applications of the invention. The term "therapeutically effective amount" refers to that amount  
5 of a receptor agonist antibody, which is useful for alleviating a selected condition. Also provided are compositions and methods for inhibiting receptor activity in order to ameliorate an undesired response.

The receptor agonist antibodies of the invention can be formulated into pharmaceutical compositions and administered in the same manner as described for  
10 mature proteins [see, e.g., International Patent Application, Publication No. WO 90/02762 (Mar. 22 1990)]. These therapeutic compositions of the invention may be administered to mimic the effect of the normal receptor ligand. These compositions may contain a pharmaceutically acceptable carrier. Suitable carriers are well known to those of skill in the art and include, for example, saline. Alternatively, such  
15 compositions may include conventional delivery systems into which protein of the invention is incorporated. Optionally, these compositions may contain other active ingredients, e.g., chemotherapeutics.

Thus, the invention also provides improved methods of treating a variety of disorders in which receptor/ligand interactions are implicated, e.g., inflammation,  
20 autoimmune disorders, etc. For example, an agonist antibody developed to a dimeric EpoR of this invention can be employed to treat acute renal failure, anemia, AIDS, and any disorder which the ligand erythropoietin can be used for treatment, e.g., for cancer chemotherapy. Similarly, an agonist antibody developed to a dimeric TpoR receptor may be employed to treat conditions of low platelet count, i.e.,  
25 patients undergoing chemotherapy for cancer, etc. An agonist antibody developed to the G-CSF dimeric receptor of this invention is useful to stimulate the polymorphonuclear cells, thereby for the treatment of conditions characterized by neutropenia, e.g., cancer chemotherapy, etc.

The invention encompasses methods of administering therapeutically  
30 effective amounts of a antibody or pharmaceutical composition of the invention to a patient. The dose, timing and mode of administration of these therapeutic or gene

therapy compositions may be determined by one of skill in the art, and may be less than or equal to the amounts of the ligand known to be administered for similar conditions. Such factors as the disease being treated, the age, physical condition, and the level of the receptor detected by the diagnostic methods described above, may be taken into account in determining the dose, timing and mode of administration of the therapeutic compositions of the invention. Generally, where treatment of an existing disorder is indicated, a therapeutic composition of the invention is preferably administered in a site-directed manner and is repeated as needed. Such therapy may be administered in conjunction with conventional therapies for such conditions.

Generally, an agonist antibody of the invention is administered in an amount between about 0.01 ng/kg body weight to about 1 g/kg and preferably about 0.01 ng/kg to 100 mg/kg per dose. Preferably, these pharmaceutical compositions are administered to human or other mammalian subjects by injection. However, administration may be by any appropriate internal route, and may be repeated as needed, e.g., as frequently as one to three times daily for between 1 day to about three weeks to once per week or once biweekly. Preferably, the agonist antibody is administered less frequently than is the ligand, when it is used therapeutically.

Optionally, the pharmaceutical compositions of the invention may contain other active ingredients or be administered in conjunction with other therapeutics. Suitable optional ingredients or other therapeutics include those conventional for treating conditions of this nature, e.g. other anti-inflammatories, diuretics, and immune suppressants, among others.

According to the methods of this invention, and as described in detail in the following examples, antibodies were generated to the erythropoietin receptor (EpoR), which mimic the agonist properties of erythropoietin. The following examples illustrate the construction and expression of exemplary multimeric receptor proteins of the invention. These examples are illustrative only and do not limit the scope of the invention.

30



Example 1 - Multimeric EpoR Antigen

A dimeric antigen of this invention was designed by fusing the extracellular domain of the erythropoietin receptor via an amino acid linker to the Fc portion of a human immunoglobulin, and expressing the fusion protein in a suitable host cell.

5 Using the following primers sets based on the published nucleotide sequence of EpoR (Jones *et al.*, Blood, 76: 31-35 (1990)), the extracellular domain of the EpoR was amplified via PCR from a human fetal liver cDNA library (Clontech).

One primer was selected from: 5' GT ATC ATG GAC CAC CTC GGG GCG TCC CTC TGG CCC CAG 3' [SEQ ID NO: 4] and 5' ATG GAC CAC CTC GGG GCG TCC CTC TGG CCC CAG 3' [SEQ ID NO: 5]. A second primer was selected from: 3' GGA GAC GGG GGG TCG ATA CAC CGA ACG AGA ATC CTG TG 5' [SEQ ID NO: 6] and 3' GGA GAC GGG GGG TCG ATA CAC CGA ACG AGA ATC 5' [SEQ ID NO: 7]; 3' CAC AGC GAC GAC TGC GGA TCG CTG GAC CTG GGG atc act ga 5' [SEQ ID NO: 8] and 3' CAC AGC GAC GAC TGC GGA TCG CTG GAC CTG GGG gtc 5' [SEQ ID NO: 9].

The resulting 250 bp DNA fragment so isolated was cloned into the vector PCR2000 (Invitrogen) and sequenced. The EpoR fragment was found to encode amino acids 1-250 [SEQ ID NO: 2] of the extracellular domain of the human EpoR [Jones *et al.*, cited above; SWISSPROT Accession number P19235].

An SpeI/XbaI fragment was isolated from the PCR2000-derived vector by digestion containing this EpoR insert, nucleotide 898 to 1647 of SEQ ID NO: 1. The insert was then cloned into the *Drosophila* S2 vector mtl [see, European Patent No. 290,261 B, published November 9, 1988] at the equivalent linker sites, SpeI and XbaI. The resulting plasmid vector, pS2EpoR, contains the EpoR extracellular domain gene insert under the control of the *Drosophila* copper metallothionein (mtn) promoter [Johansen *et al.*, Genes and Development, 3:882-889 (1989); Angelichio *et al.*, Nucl. Acid Res., 19:5037-5043 (1991)].

The plasmid pS2EpoR was digested with BssH2 and XbaI and a C terminal fragment of the EpoR sequence was removed by this digestion. A plasmid

containing the whole EpoR gene fused to a bridging moiety was then prepared by cloning the following three fragments:

- (1) the large BssH2/XbaI fragment from the above digestion;
- (2) a synthetic BssH2/KpnI linker, which spanned nucleotide 1561 to nucleotide 1659 of SEQ ID NO: 1, which encoded the C terminus of the EpoR extracellular domain, aa 222 to 250 of SEQ ID NO: 2, linked in frame to the four amino acid recognition sequence for protease Factor Xa cleavage (IleGluGlyArg) [amino acids 251 to 254 of SEQ ID NO: 2], and
- (3) a KpnI/XbaI fragment containing the human IgG1 Fc region, spanning nucleotide 1660 to the XbaI site which appears at nucleotides 2371-2376 of SEQ ID NO: 1 [see, also Johansen *et al.*, J. Biol. Chem., 270:9459-9471 (1995)]. This KpnI/XbaI fragment was constructed as follows:

Human IgG1 cDNA encoding CH1, the hinge, CH2 and CH3 described by J. Ellison *et al.*, Nucleic Acids Res., 10: 4071-4079 (1982) was cloned from the human IgG plasma cell leukemia ARH-77 (American Type Tissue Collection), using RT-PCR. This cDNA was fully sequenced to confirm identity with the published sequence [see, International patent publication WO 92/00985]. This sequence was inserted into a pUC18 vector (pUC18-Fc). This vector was digested with KpnI and SacII, deleting the CH1, hinge and part of CH2. The deleted region was replaced with a PCR amplified fragment containing the hinge-CH2 region as follows.

Using the following PCR primers: 5' TCG AGC TCG GTA CCG AGC CCA AAT CGG CCG ACA AAA CTC ACA C 3' [SEQ ID NO: 10] and 5' GTA CTG CTC CTC CCG CGG CTT TGT CTT G 3' [SEQ ID NO: 11], a DNA fragment containing the hinge-CH2 region was amplified from pUC18-Fc, digested with KpnI and SacII, gel purified and cloned back into the KpnI/SacII digested pUC18-Fc vector. The Cys, which occurs at position 230 [Kabat numbering; Kabat *et al.*, "Sequences of Proteins of Immunological Interest, 5th Edition, US Department of Health and Human Services, NIH Publication No. 91-3242 (1991); this is also residue 5 of the hinge of the IgG1 heavy chain; residue 261 of SEQ ID NO: 2] was altered to an Ala through a TGT to GCC substitution in the nucleotide sequence to

avoid having the unpaired Cys present which is usually involved in light chain-heavy chain crosslinking.

An altered DNA sequence in one of the PCR primers introduced a unique KpnI site at the 5' end of the hinge. The resulting plasmid was called pUC18Fcmod, and the junctions and PCR amplified region were sequenced for confirmation.

The entire hinge-CH2-CH3 insert in pUC18-Fcmod was removed in a single DNA fragment with KpnI and XbaI, gel purified, and ligated into SFcR1Cos4 cut with KpnI and XbaI to create COSFc. SFcR1Cos4 is a derivative of pST4DHFR [K. Deen *et al*, Nature, 331: 82 (1988)] and contains the soluble Fc receptor type I (sFcR1) inserted between the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation regions, and also contains the dihydrofolate reductase (DHFR) cDNA inserted between the b-globin promoter and SV40 polyadenylation regions, an SV40 origin of replication, and an ampicillin resistance gene for growth in bacteria.

Cutting the vector with KpnI and XbaI removes the sFcR1 coding region, so that the COSFc vector contains the hinge-CH2-CH3 region inserted between the CMV promoter and BGH polyA regions. The COSFcLink vector was made from COSFc by inserting an oligonucleotide linker at the unique EcoRI site of the vector, which recreates this EcoRI site, and also introduces BstEII, PstI and EcoRV cloning sites. The oligonucleotides used were:

5' AATTCGGTTACCTGCAGATATCAAGCT 3' [SEQ ID NO: 12] and  
3' GCCAATGGACGTCTATAGTTCGATTAA 5' [SEQ ID NO: 13]. The junction was sequenced to confirm orientation in the vector. The size of the final vector is 6.37 kb and is reported as SEQ ID NO: 3. The KpnI/XbaI fragment used in the dimeric immunogen described herein was obtained from COSFcLink.

The resulting plasmid DNA construct containing the three fragments described above was called pmtalsEpoRfc [SEQ ID NOS: 1 and 2]. The plasmid sequence contains the heterologous fusion sequence comprising an intact EpoR extracellular domain encoding amino acids 1-250 of SEQ ID NO: 2 linked via a four amino acid linker (aa 251-254 of SEQ ID NO: 2) to a human IgG1 Fc region (aa 255

to 488 of SEQ ID NO: 2). In the plasmid, the fusion sequence was under the control of the mtn promoter, described above.

Plasmid pmtalsEpoFc was cotransfected into *Drosophila* S2 cells with a vector encoding hygromycin resistance [see, EP No. 290,261B, cited above]. Stable  
5 co-transfectants were selected in hygromycin, and expression of the EpoR induced by Cu<sub>2</sub>SO<sub>4</sub> according to published protocols [Johansen *et al.*, cited above; Angelichio *et al.*, cited above].

The co-transfected cells secreted the EpoRFc protein as a dimeric molecule due to the natural affinity of the Fc sequence for itself. Under reducing conditions in  
10 SDS-PAGE, the EpoRFc protein ran as a monomer. The dimeric protein was purified from *Drosophila* medium by passage over a Protein A Sepharose column.

To obtain the monomeric EpoR extracellular protein apart from its Fc fusion, the EpoRFc fusion protein is treated as follows: EpoR-Fc was dialyzed into 20 mM Tris, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, pH 8. Factor Xa (New England Biolabs) was  
15 added at a ratio of 1 mg Factor Xa per 25 mg EpoR-Fc and incubated at 6°C for 18-20 hours.

The digest was then added to Protein A Sepharose 4 Fast Flow [Pharmacia], washed with 100 mM Tris, pH 8, giving a ratio of 0.5 ml packed resin per mg protein. Following a 90 minute incubation with mixing, at 6°C, the resin was  
20 separated from the supernatant by centrifugation. SDS/PAGE and Western blots showed that EpoR, free of Fc, was present in the supernatant. The N-terminal sequence for EpoR was correct. The final product was sterile filtered.

#### Example 2 - Erythropoietin Binding Assay

25 The activity of the dimeric fusion protein EpoRFc and the monomeric single-stranded mEpoR protein cleaved from EpoRFc were tested in various biological assays by their ability to neutralize the activity of erythropoietin (Epo).

##### *A. Inhibition of Epo-induced proliferation of UT7-Epo Cells*

An assay for the measurement of Epo activity on the proliferation of UT7Epo  
30 cells, which are dependent on Epo for growth [Komatsu *et al.*, Blood, 82:456-464 (1993)] was performed as follows. The Epo used in the experiment is Epogen (2000

U/ml) [Amgen, Thousand Oaks, Ca], diluted in phosphate buffered saline (PBS) and human serum albumin (HSA) for storage at 4°C at 200 U/ml. Dilutions of cleaved EpoR or EpoRFc protein samples were made, so that final concentrations ranged from 0.001 to 100 ng/ml.

5        Samples of either EpoR or EpoRFc were added to wells at 10 ul/well in quadruplicate. Epo (0.2 U/ml) was added to each well. UT7Epo cells ( $1 \times 10^5$  cells/ml) were plated at 100 ul/well. After the plates were incubated at 37°C for three days, 10 ul/well of  $^3\text{H}$ -thymidine (diluted to 100 mCi/ml in IMDM+10% fetal calf serum (FCS)) were added to a final concentration of 10 uCi/ml. Plates were  
10    incubated at 37°C for four hours with  $^3\text{H}$ -thymidine. The 96-well plates were harvested onto glass fiber filters using the Tomtec plate harvester with 10% cold TCA and cold 95% ethanol. Solid scintillant was melted onto the filters and the samples counted. The mean and standard error of quadruplicate samples was determined.

15        The data were reported as the percent of positive (0.2 U/ml) Epo control and are illustrated in Fig. 1.

*B. Inhibition of Epo-induced proliferation of 32D/Epo wt Cells*

Another assay was performed for the measurement of Epo activity on the proliferation of 32D/Epo wt cells (Miura, O. *et al.*, Mol. Cell. Biol., 13:1788-1795  
20    (1993)). 32D/Epo wt is an IL-3 dependent cell line transfected with the human Epo receptor.

This assay is performed as described in Part A above, with the modification that the each well contains 1 U/ml Epo. The results of this assay are illustrated in Fig. 2.

25        *C. Inhibition of Epo-induced CFU-E colony formation*

A third assay for measuring the ability of Epo to stimulate the differentiation of murine bone marrow cells to hemoglobin producing mature erythrocytes (CFU-E assay) was performed as described below.

Murine bone marrow cells were flushed from the femur of female B2D6F1  
30    mice. The marrow cells ( $1 \times 10^5$  cells/ml final concentration) were mixed with IMDM, 25% FCS (final) and methylcellulose (0.8 % final). 0.4 ml cells was plated

per well of 24-well TC plate. EpoRFc (40 ul/well) samples were added and Epo (1 U/ml) was added to each well. Plates were incubated at 37°C, 5% CO<sub>2</sub>, 6% O<sub>2</sub> for two days.

CFU-E colonies containing 8 or more red cells were counted. The mean and standard error of triplicate samples were determined and data reported as the percent of the positive (1 U/ml) Epo control. The results of this assay are illustrated in Fig. 3.

In all three assays, both the monomeric extracellular domain protein EpoR and the dimeric fusion protein EpoRFc were able to bind, and thereby neutralize, the biological activity of the ligand Epo. However, the dimeric fusion protein EpoRFc was consistently 10 to 100 fold more effective at neutralizing Epo activity than non-fused, monomeric extracellular domain protein EpoR, suggesting that the dimeric antigen had a higher affinity to the natural ligand, Epo. The assay results also confirm that the dimeric fusion protein mimics the cell surface form of the natural EpoR in being able to bind to Epo with greater affinity than the mEpoR domain protein.

### Example 3 - Agonist Antibodies of the Invention

#### *A. Generation of Hybridomas*

Mice were immunised s.c. with recombinant EpoRFc (34ug) in Freund's complete adjuvant and then boosted i.p. 4 weeks later (34ug) with Freund's incomplete adjuvant. One and three days before fusion mice received 20ug in PBS i.p.. The spleens were harvested and fused with myeloma cells according to the method described in Zola. ( Monoclonal Antibodies : A Manual of Techniques, Zola H. ed., Boca Raton, Fl : CRC Press, 1987)

Positive hybridomas were selected through a primary screen described below. Positives were rescreened using a competitive immunoassay and then BIAcore was used to select hybridomas that expressed high affinity monoclonal antibodies which were then cloned twice by the limiting dilution method.

A1. Primary Screening assay of anti EpoRFc Hybridomas

96 well microtitre plates were coated with 100ul/well of EpoRFc at 0.5ug/ml in coating buffer (50mM Na<sub>2</sub>HPO<sub>4</sub>, 150mM NaCl, 0.02% v/v Kathon, pH 7.4) and incubated overnight at 4°C. The wells were aspirated and 250ul/well blocking buffer  
5 (1% w/v BSA, 50mM Tris, 150mM NaCl, 0.1% v/v Kathon, pH 7.4) added for 1 hour at 37°C. The wells were washed X4 with wash buffer (10mM Tris, 150mM NaCl, 0.05% Tween 20, pH 7.4) and 50ul/well of 40ug/ml human IgG diluted in assay buffer (0.5% w/v BSA, 0.05% w/v bovine γ globulin, 50mM Tris, 150mM NaCl, 7.86mg/L DTPA, 0.1g/L Tween-40, 0.02% v/v Kathon, pH 7.4) added  
10 followed by 50ul hybridoma supernatant. The plates were incubated for 1 hour at 37°C on a plate shaker, the wells washed X4 and then 100ul/well Europium conjugated anti-mouse IgG added (0.5ug/ml in assay buffer). After incubation for 1 hour at 37°C on the plate shaker the wells were again washed X4 and 100ul/well of enhancement solution added into each well and incubated for 2 min at 22C on the  
15 plate shaker and the counts read on a Delfia plate reader.

A2. Isolation of Hybridomas Producing High Affinity Antibodies that Recognise Solution Phase EpoRFc using a competitive immunoassay.

Microtitre plates were coated with EpoRFc and blocked as above. The wells  
20 were washed X4 and then 50ul of either EpoRec at 6ug/ml diluted in assay buffer or 50ul human IgG at 40ug/ml diluted in assay buffer or 50ul assay buffer alone were added followed by 50ul hybridoma supernatant. After incubation for 1 hour at 37°C on the plate shaker the wells were washed X4 followed by addition of 100ul/well Europium conjugated anti-mouse IgG at 0.5ug/ml (diluted in assay buffer). After  
25 incubation for 1 hour at 37°C on the plate shaker the wells were again washed X4 and 100ul/well of enhancement solution added into each well and incubated for 2 min at 22°C on the plate shaker and the counts read on a Delfia plate reader.

Positive antibodies would be displaced by solution phase EpoRFc and these would thus show a reduction in counts when compared to wells with only assay  
30 buffer, antibodies giving a reduction in counts with human IgG would be non specific. High affinity antibodies would show >80% reduction in counts.

A hybridoma designated herein as 1C8 (or alternatively as 5-1C8) has been deposited at the European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wiltshire, SP4 OJG United Kingdom on 5 June 1996 and assigned provisional Accession number 96060519. The deposit referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited material, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited material, and no such license is hereby granted.

15        A3.    Binding Analysis in the BIAcore for the Selection of Antibodies that Bound EpoR with High Affinity

Rabbit anti-mouse Fc (RAMFc) was immobilised to the sensor chip surface (Pharmacia Biosensor protocol) and used as a capture antibody. The run buffer used was 10mM HEPES, 150mM NaCl, 3.4mM EDTA, 0.005% (v/v) Surfactant P 20 at a flow rate of 5ul/min. 20ul hybridoma supernatant was injected over the sensor chip surface followed by 20ul EpoR or EpoRfc (3ug/ml diluted in run buffer). The surface was regenerated by injection of 15ul HCL (100mM) and then 10ul SDS (0.025%).

High affinity monoclonal antibodies were selected on the basis of a fast on rate and slow off rate with respect to EpoR binding.

25        B.     Assays to Test Agonist Characteristic of Antibodies

The hybridoma supernatants or purified antibodies were then tested for their ability to bind to the naturally occurring EpoR on UT7 or 32D/Epo wt cells in flow cytometry as follows.  $5 \times 10^5$  cells per sample of 32D/Epo wt or UT7-Epo cells were resuspended in 50 ul PBS/10%BSA. 5.0 ul of each purified anti-EpoRfc was



diluted to 30ug/ml and incubated on ice for 45 minutes. Cells were washed and resuspended in 50 ul PBS/10% bovine serum albumin (BSA) and 7 ul FITC-labelled goat anti-mouse IgG (Fab')<sub>2</sub> [Tago] added for 45 minutes on ice. Cells were again washed in PBS/10%BSA and resuspended in 0.4 ml PBS/10% BSA and then 0.2 ml  
5 3.2% paraformaldehyde added followed by vortexing. Cells were stored at 4°C until analysis on Becton-Dickinson's FACScan fluorescence activated cell sorter.

The data, illustrated in Figure 4, was reported as the % of control antibody fluorescence. As can be seen, four antibodies stained UT7Epo cells while three did not. Interestingly, only one of the four positive antibodies on UT7Epo cells was able  
10 to recognize the transfected human Epo receptor in 32D/Epo wt cells, suggesting some differences in the disposition of the Epo receptor in these cell lines. This also suggests differences in the epitopes recognized by these antibodies.

C. Assays to Test agonist activity of antibodies.

15 (1) Hybridoma supernatants or purified antibodies were tested for their ability to mimic the activity of Epo by stimulating the proliferation of UT7-Epo cells in an assay performed as described in Example 2A above, with the modification that Epo is absent from wells containing the hybridoma supernatants. The only sample was dilutions of the monoclonal antibodies against EpoR<sub>Fc</sub> dimeric protein. Results  
20 of this assay are shown in Figure 5 for some purified EpoR reactive monoclonal antibodies. Four of the antibodies gave significant proliferative activity, in one case approaching that of Epo itself (1C8). The variability of the extent of peak activity relative to Epo suggests that the way in which the antibodies bind may be an important determinant of activity.

25 (2) Hybridoma supernatants or purified antibodies were tested for their ability to mimic the activity of Epo by stimulating the proliferation and differentiation of human bone marrow progenitor cells to form red blood cell colonies (CFU-E) similar to the assay described above in Figure 6 . The present assay differed in that the progenitor cells were of human origin.

30 In this procedure, light density cells from human bone marrow centrifuged over Histopaque 1077 were washed and resuspended at  $2.5 \times 10^6$  cells/ml in X-vivo

medium (BioWhittaker). The purified monoclonal antibodies were diluted in X-vivo medium, and the Epo positive control was 4 U/ml. For the assay, 0.3 ml cells, 0.3 ml Mab sample (or Epo control) and 0.7 ml X-vivo medium were incubated in a polypropylene tube for 30 min at RT, then 0.9 ml FCS, 0.3 ml 10% BSA and 0.8 ml 3.2% methylcellulose were added. 0.4 ml were plated per well of a 24-well TC dish (Nunc). This procedure departs from the standard assay in the pre-incubation of cells, X-Vivo and Mab alone for 30 min (without serum, BSA or methylcellulose) prior to plating in methylcellulose.

The results are shown in Figure 6. Once again, all four antibodies were positive in the UT7Epo proliferation assay were also able to stimulate the generation of mature red blood cells. Again the most potent was antibody 1C8 which gave almost 50% of the maximal activity of human Epo on the same cells. The order of maximal activity was similar to that observed in the proliferative assay.

**D. Epitope mapping of agonist antibodies.**

It was likely that one contributor to the different activities of the agonist antibodies was the epitope recognized on the erythropoietin receptor. To determine if the antibodies recognized overlapping epitopes on the Epo receptor, the ability of antibodies to compete with each other in binding to EpoR or EpoRFc was measured. RAMFc was immobilised to the sensor chip surface and using a flow rate of 5ul/min the following sequential injections were used. 5ul of first monoclonal antibody (25 or 30ug/ml), 10ul EpoR (5ug/ml) or EpoRFc (6ug/ml), 2 X 5ul of nonspecific monoclonal antibodies, (100ug/ml each of IgG 2bk, IgG 3k, IgG 1k and IgG 2ak), 5ul of second monoclonal antibody. The surface was regenerated with 15ul 0.1M phosphoric acid and 8ul 0.025% SDS at 10ul/min. The data are shown in Table I.

**Table I** Competition of different monoclonal antibodies for epitopes on Epo receptor as measured by BIAcore. Results are expressed as Response Units. The antibody attached to the chip via protein A is shown in the left hand column and

each row shows the binding of each monoclonal after prebinding of EpoR or EpoRFc.

<u>Epo-R</u>	Measurements are in RU		
	5-1C8	3-2B6	5-2G6
5-1C8	-25	-21	2
3-2B6	-3	-6	269
5-2G6	-13	169	-
<u>Epo- RFc</u>	Measurements are in RU		
	5-1C8	3-2B6	5-2G6
5-1C8	-9.6	6.3	5.2
3-2B6	-7	-8	272
5-2G6	-11.6	167	-5

5

The data indicate that of the three agonist antibodies, 2B6 and 2G6 bind to two non-overlapping epitopes since they do not compete for binding to the Epo receptor. In contrast, 1C8 competes with both 2B6 and 2G6, suggesting that it binds to a third distinct epitope which overlaps the other two. Thus there is no single epitope which can lead to agonist activity, but the precise epitope can very likely affect the extent of bioactivity observed. Use of the EpoRFc as antigen seems to generate a wide variety of agonist epitopes.

#### 15 *E. Competition of antibody binding with Epo.*

Another measure of differences between the agonist antibodies is provided by studies which measure the ability of monoclonal antibody to compete with Epo binding to the receptor. These experiments were conducted in two ways. First, a goat anti-human IgG was attached to the BIAcore chip, followed by sequential binding of EpoRFc, Epo and finally monoclonal antibody. In this experiment, pre-binding of Epo blocked the binding of 1C8 by more than 75%, but had only a limited effect on the binding of 1D9, 2G6 and 2B6. Second, if the EpoRFc binding to the chip was followed first by monoclonal antibody and then by Epo, all four monoclonal antibodies could block subsequent binding of Epo. More specifically, a goat anti-human IgG, Fc specific antibody was immobilised on the sensor chip surface.

25

- Injection of 25ul EpoRFc (2ug/ml) at 5ul/min was followed by injection of 25ul Epo (5ug/ml) then 25ul Mab (10ug/ml) at 5ul/min. RU recorded. The surface was regenerated with injections of 15ul 0.1M phosphoric acid (5ul/min) and 8ul 0.025% SDS at 10ul/min. and as mentioned above the experiment was repeated reversing the order of addition for Epo and mAb, i.e inject Mab first, then displaced with Epo.

These data, shown in Table II suggest that all four antibodies may block access of Epo to its binding site, but only the 1C8 antibody overlaps substantially with the Epo binding site on the Epo receptor.

- Table II Competition of monoclonal antibody binding with Epo binding to Epo receptor as measured by BIAcore. The order of addition is left to right as indicated. The identity of each monoclonal antibody is listed in the left column.

<b>EpoRFc + Epo + Mab</b>			
Mab	EpoR R.U.	Epo R.U.	Mab R.U.
Buffer	557	118	-9.5
1D9	475	106	173
2B6	471	106	264
2G6	468	104	300
1C8	465	104	77
<b>EpoR + Mab + Epo</b>			
Mab	EpoR R.U.	Mab R.U.	Epo R.U.
Buffer	462	9.1	105
1D9	452	173	101
2B6	449	455	9.2
2G6	448	474	25
1C8	447	436	-196

15

#### *F. Affinity of the monoclonal antibodies to the Epo receptor*

- The binding kinetics and affinities of the agonist antibodies was determined by measurements in the BIAcore with both the soluble EpoR and with the dimeric EpoRFc. Specifically, RAMFc was immobilised to the sensor chip surface and a flow rate of 5ul/min was used with run buffer. The Mab was first bound to the RAMFc (5ul injection) followed by a 20ul injection of of EpoR (0-4ug/ml) or EpoRFc (0-6ug/ml) then buffer flow for 120 sec and regeneration with 15ul 0.1M phosphoric acid and 8ul 0.025% SDS at 10ul/min.

The four antibodies showed quite different kinetics and binding constants as listed in Table III..

Table III Affinities and kinetics of binding of monoclonal antibodies to Epo  
5 receptor as measured by BIAcore.

<b>Binding to Epo-R</b>					
<b>Mab</b>	<b>K<sub>ass</sub>. M<sup>-1</sup> s<sup>-1</sup></b>	<b>K<sub>diss</sub>. (s)</b>	<b>K<sub>D</sub> (M)</b>	<b>(2nd Dissoc. K<sub>D</sub>)</b>	
<b>1C8</b>	$2.54 \times 10^5$	$5.9 \times 10^{-3}$	$2.3 \times 10^{-8}$	$4.3 \times 10^{-9}$	
<b>2G6</b>	$1.64 \times 10^5$	$4.3 \times 10^{-4}$	$2.6 \times 10^{-9}$		
<b>2B6</b>	$2.4 \times 10^5$	$1.9 \times 10^{-4}$	$7.8 \times 10^{-10}$		
<b>Binding to Epo-RFc</b>					
<b>Mab</b>	<b>K<sub>ass</sub>. M<sup>-1</sup> s<sup>-1</sup></b>	<b>K<sub>diss</sub>. (s)</b>	<b>K<sub>D</sub> (M)</b>		
<b>1C8</b>	$4 \times 10^5$	N/D	$< 5 \times 10^{-10}$		
<b>2G6</b>	$1.77 \times 10^5$	N/D	$< 5 \times 10^{-10}$		
<b>2B6</b>	$1.96 \times 10^5$	N/D	$< 5 \times 10^{-10}$		
<b>N/D No Dissociation</b>					

10 On the monomeric EpoR, the affinities ranged between 0.75nM and 23nM. Interestingly, the most biologically potent antibody, 1C8, had the lowest dissociation constant of 23nM , and also had a second binding mode with a dissociation constant of 4.3nM. It was the only antibody to show this phenomenon. In contrast, with the dimeric receptor EpoRFc, all four antibodies had non-measurable dissociations,  
15 suggesting dissociation constants of less than 500pM. These data suggest that kinetics may also play a role in the relative agonist activity of different monoclonal antibodies.

All documents cited above are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present  
5 invention are believed to be encompassed in the scope of the claims appended hereto.

(1) GENERAL INFORMATION:

- (i) APPLICANT: Young, Peter R.  
Erickson-Miller, Connie
- (ii) TITLE OF INVENTION: Method for Obtaining Receptor Agonist  
Antibodies
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: SmithKline Beecham Corporation- Corporate  
Patents
  - (B) STREET: 709 Swedeland Road
  - (C) CITY: King of Prussia
  - (D) STATE: Pennsylvania
  - (E) COUNTRY: USA
  - (F) ZIP: 19406-2799
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Jervis, Herbert H.
  - (B) REGISTRATION NUMBER: 31,171
  - (C) REFERENCE/DOCKET NUMBER: SBC P50349-1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 610-270-5015
  - (B) TELEFAX: 610-270-5090

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4990 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 898..2361

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC	180
ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC	240
ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT	300
TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT	360
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GTTGTGGTCA GCAGCAAAAT CAAGTGAATC ATCTCAGTGC AACTAAAGGG GGGATCCGAT	840



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Met Asp His Leu Gly Ala Ser Leu Trp Pro Gln Val Gly Ser Leu Cys	
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Pro Lys Phe Glu Ser Lys Ala Ala Leu Leu Ala Ala Arg Gly Pro Glu	
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GAG CTT CTG TGC TTC ACC GAG CGG TTG GAG GAC TTG GTG TGT TTC TGG	1089
Glu Leu Leu Cys Phe Thr Glu Arg Leu Glu Asp Leu Val Cys Phe Trp	
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Tyr Gln Leu Glu Asp Glu Pro Trp Lys Leu Cys Arg Leu His Gln Ala	
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370 375 380	
CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC	2097
Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr	
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Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser	
405 410 415	
GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC	2193
Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr	
420 425 430	
AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC	2241
Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr	
435 440 445	
AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC	2289
Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe	
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CAAACCACAA CTAGAATGCA GTGAAAAAA TGCTTTATTT GTGAAATTTG TGATGCTATT	2511

GCTTTATTTG TAACCATTAT AAGCTGCAAT AAACAAGTTA ACAACAACAA TTGCATTTCAT 2571

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AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC GTTGTGCCA TTGCTGCAGG 4251

CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC AGCTCCGGTT CCCAACGATC 4311

AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAGCG GTTAGCTCCT TCGGTCTCTC 4371

GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG CAGCACTGCA 4431

TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG AGTACTCAAC 4491

CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGCCCGG CGTCAACACG 4551

GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCATTGGAA AACGTTCTTC 4611

GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCTCTCG 4671

TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTACCAGC GTTCTGGGT GAGCAAAAAC 4731

AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT GAATACTCAT 4791

ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA TGAGCGGATA 4851

CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT TTCCCCGAAA 4911

AGTGCCACCT GACGTCTAAG AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG 4971

TATCACGAGG CCCTTTCGT 4990

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 488 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp His Leu Gly Ala Ser Leu Trp Pro Gln Val Gly Ser Leu Cys  
 1 5 10 15  
 Leu Leu Leu Ala Gly Ala Ala Trp Ala Pro Pro Pro Asn Leu Pro Asp  
 20 25 30  
 Pro Lys Phe Glu Ser Lys Ala Ala Leu Leu Ala Ala Arg Gly Pro Glu  
 35 40 45  
 Glu Leu Leu Cys Phe Thr Glu Arg Leu Glu Asp Leu Val Cys Phe Trp  
 50 55 60  
 Glu Glu Ala Ala Ser Ala Gly Val Gly Pro Gly Asn Tyr Ser Phe Ser  
 65 70 75 80  
 Tyr Gln Leu Glu Asp Glu Pro Trp Lys Leu Cys Arg Leu His Gln Ala  
 85 90 95  
 Pro Thr Ala Arg Gly Ala Val Arg Phe Trp Cys Ser Leu Pro Thr Ala  
 100 105 110  
 Asp Thr Ser Ser Phe Val Pro Leu Glu Leu Arg Val Thr Ala Ala Ser  
 115 120 125  
 Gly Ala Pro Arg Tyr His Arg Val Ile His Ile Asn Glu Val Val Leu  
 130 135 140  
 Leu Asp Ala Pro Val Gly Leu Val Ala Arg Leu Ala Asp Glu Ser Gly  
 145 150 155 160  
 His Val Val Leu Arg Trp Leu Pro Pro Pro Glu Thr Pro Met Thr Ser  
 165 170 175

His Ile Arg Tyr Glu Val Asp Val Ser Ala Gly Asn Gly Ala Gly Ser  
 180 185 190

Val Gln Arg Val Glu Ile Leu Glu Gly Arg Thr Glu Cys Val Leu Ser  
 195 200 205

Asn Leu Arg Gly Arg Thr Arg Tyr Thr Phe Ala Val Arg Ala Arg Met  
 210 215 220

Ala Glu Pro Ser Phe Gly Gly Phe Trp Ser Ala Trp Ser Glu Pro Val  
 225 230 235 240

Ser Leu Leu Thr Pro Ser Asp Leu Asp Pro Ile Glu Gly Arg Gly Thr  
 245 250 255

Glu Pro Lys Ser Ala Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala  
 260 265 270

Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro  
 275 280 285

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val  
 290 295 300

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val  
 305 310 315 320

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln  
 325 330 335

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln  
 340 345 350

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala  
 355 360 365

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro  
 370 375 380

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr  
 385 390 395 400

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser  
 405 410 415

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr  
 420 425 430

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr  
 435 440 445

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe  
 450 455 460

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys  
 465 470 475 480

Ser Leu Ser Leu Ser Pro Gly Lys  
 485

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6367 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ACGGTAAATG GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTTGAC GTCAATAATG	180
ACGTATGTTT CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG GGTGGACTAT	240
TTACGGTAAA CTGCCCACTT GGCAGTACAT CAAGTGTATC ATATGCCAAG TACGCCCCCT	300
ATTGACGTCA ATGACGGTAA ATGGCCCGCC TGGCATTATG CCCAGTACAT GACCTTATGG	360



GACTTTCCTA CTTGGCAGTA CATCTACGTA TTAGTCATCG CTATTACCAT GGTGATGCGG	420
TTTTGGCAGT ACATCAATGG GCGTGGATAG CGGTTTGACT CACGGGGATT TCCAAGTCTC	480
CACCCCATTG ACGTCAATGG GAGTTTGTTT TGGCACCAAA ATCAACGGGA CTTTCCAAAA	540
TGTCGTAACA ACTCCGCCCC ATTGACGCAA ATGGGCGGTA GGC GTGTACG GTGGGAGGTC	600
TATATAAGCA GAGCTGGGTA CGTGAACCGT CAGATCGCCT GGAGACGCCA TCGAATTCCG	660
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ATGCCACCG TGCCACGAC CTGAACTCCT GGGGGGACCG TCAGTCTTCC TCTTCCCCC	780
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CGTGAGCCAC GAAGACCCTG AGGTCAAGTT CAACTGGTAC GTGGACGGCG TGGAGGTGCA	900
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CAAAGCCCTC CCAGCCCCCA TCGAGAAAAC CATCTCCAA GCCAAAGGGC AGCCCCGAGA	1080
ACCACAGGTG TACACCCTGC CCCCATCCCG GGATGAGCTG ACCAAGAACC AGGTCAGCCT	1140
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TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGATCA ACGATAGCTT ATCTGTGGGC	2940
GATGCCAAGC ACCTGGATGC TGTGGTTTC CTGCTACTGA TTAGAAGCC ATTTGCCCCC	3000

TGAGTGGGGC TTGGGAGCAC TAACTTTCTC TTTCAAAGGA AGCAATGCAG AAAGAAAAGC	3060
ATACAAAGTA TAAGCTGCCA TGTAATAATG GAAGAAGATA AGGTGTATG AATTAGATTT	3120
ACATACTTCT GAATTGAAAC TAAACACCTT TAAATTCCTA AATATATAAC ACATTTTCATA	3180
TGAAAGTATT TTACATAAGT AACTCAGATA CATAGAAAAC AAAGCTAATG ATAGGTGTCC	3240
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TCTTCAACGA ATTAGAAAGA GCAATCTGCA AACTCATCTG GAATAACAAA AAACCTAGGA	3360
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AGCTGTACTA CAGAGCAATT GTGATAAAAA CTGCATGGTA CTGATATAGA AACGGACAAG	3480
TAGACCAATG GAATAGAACC CACACACCTA TGGTCACTTG ATCTTCAACA AGAGAGCTAA	3540
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CTTCCTTTTT CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA GCGGATACAT	6300
ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTT CCCGAAAAGT	6360
GCCACCT	6367

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTATCATGGA CCACCTCGGG GCGTCCCTCT GGCCCCAG

38

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGACCACC TCGGGCGTC CCTCTGGCCC CAG

33

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTGTCCTAAG AGCAAGCCAC ATAGCTGGGG GGCAGAGG

38

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTAAGAGCAA GCCACATAGC TGGGGGGCAG AGG

33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGTCACTAGG GGTCCAGGTC GCTAGGCGTC AGCAGCGACA C

41

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGGGGGTCC AGGTCGCTAG GCGTCAGCAG CGACAC

36

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGAGCTCGG TACCGAGCCC AAATCGGCCG ACAAACCTCA CAC

43

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTACTGCTCC TCCCGCGGCT TTGTCTTG

28

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATTCGGTTA CCTGCAGATA TCAAGCT

27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATTAGCTTG ATATCTGCAG GTAACCG

27

## WHAT IS CLAIMED IS:

1. A method for generating an antibody capable of acting as an agonist of a receptor comprising: introducing into an immunocompetent animal or isolated  
5 immunocompetent cells thereof an antigenically effective amount of a recombinant immunogen comprising a first extracellular receptor domain spaced apart from a second extracellular receptor domain by a bridging moiety which places said first domain and said second domain in functional proximity which mimics the functional domain proximity of a native multimeric receptor.  
10
2. The method according to claim 1 wherein said native multimeric receptor is a homodimer and said first domain and said second domain are the same.
3. The method according to claim 1 wherein said native multimeric  
15 receptor is a heterodimer and said first domain and said second domain are different subunits of the same receptor.
4. The method according to claim 1 wherein said bridging moiety is  
peptidic.  
20
5. The method according to claim 4 wherein said bridging moiety is an amino acid amphipathic helix.
6. The method according to claim 5 wherein said helix is a leucine  
25 zipper.
7. The method according to claim 4 wherein said bridging moiety is an Fc portion of a human immunoglobulin with an intact hinge region.
- 30 8. The method according to claim 1 wherein said bridging moiety is an organic non-peptidic molecule.

9. The method according to claim 8 wherein said organic molecule is a bifunctional cross linker.

5 10. The method according to claim 9 wherein said cross-linker is selected from the group consisting of carbodiimide, glutaraldehyde, DSS, and BS3.

11. In a method for generating a monoclonal antibody comprising introducing an immunogen into an animal or to isolated cells thereof, isolating  
10 antibody producing cells therefrom, fusing said antibody producing cells with immortalized cells and isolating a hybridoma cell line which secretes a monoclonal antibody to said immunogen, the improvement comprising employing as the immunogen the immunogen of claim 1.

15 12. An antibody produced by the method of claim 1.

13. An antibody produced by the method of claim 11.

14. An altered antibody comprising a portion of the antibody selected  
20 from the group consisting of the antibody of Claim 12 and Claim 13.

15. A recombinant immunogen comprising a first extracellular receptor domain spaced apart from a second extracellular receptor domain by a bridging moiety which places said first domain and said second domain in functional  
25 proximity which mimics the functional domain proximity of a native multimeric receptor.

16. The immunogen according to claim 15 wherein said multimeric receptor is a homodimer or heterodimer.

30

17. The immunogen according to claim 15 wherein said bridging moiety is peptidic domain.

18. The immunogen according to claim 15 wherein said bridging moiety is an organic non-peptidic molecule.

19. A recombinant polynucleotide comprising a nucleotide region encoding a first extracellular domain of a receptor molecule fused in the correct reading frame to a nucleotide region encoding a peptidic bridging moiety which is fused in the correct reading frame to a nucleotide region encoding a second extracellular domain of a receptor molecule.

20. The recombinant polynucleotide according to claim 19 comprising a nucleotide region encoding a first extracellular domain of a receptor molecule fused in the correct reading frame to a nucleotide region encoding a peptidic bridging moiety.

21. The polynucleotide according to claim 19 wherein a further nucleotide region encoding an enzymatically cleavable peptide sequence is optionally inserted in the correct reading frame between: (a) the region encoding said first extracellular domain and the region encoding said bridging moiety; or (b) the region encoding the bridging moiety and the region encoding said second extracellular domain; or both (a) and (b).

22. The polynucleotide according to claim 20 wherein a further nucleotide region encoding an enzymatically cleavable peptide sequence is optionally inserted in the correct reading frame between the region encoding said first extracellular domain and the region encoding said bridging moiety.

23. A vector comprising a polynucleotide sequence of any of the claims 19-22 under the control of suitable regulatory sequences capable of directing replication and expression of said polynucleotide sequence in a host cell.

5 24. A host cell transformed with the vector according to claim 23.

25. A recombinant immunogen comprising a first extracellular receptor domain of the erythropoietin receptor spaced apart from a second erythropoietin receptor extracellular domain by an Fc region with intact hinge region of a human  
10 antibody, which places said first domain and said second domain in functional proximity which mimics the functional domain proximity of the native dimeric erythropoietin receptor.

26. The immunogen according to claim 25, which is EpoRFc.  
15

27. An EPO agonist antibody characterized as possessing at least 20% of EPO activity when measured in a CFU(E) assay or at least 40% of EPO activity when measured in a proliferation assay or having an affinity for EpoR as measured by Kd equal to or less than about 23nM, or having an affinity for EpoRFc as  
20 measured by Kd of equal to or less than 500pM.

28. The antibody according to claim 27 wherein said antibody possesses at least 40% of EPO activity when measured in a CFU(E) assay or at least 60% of EPO activity when measured in a proliferation assay.  
25

29. The antibody according to claim 27 wherein the antibody is selected from the group consisting of 1C8 and 2G6.

30. A method of treating anemia in a patient comprising administering a  
30 therapeutically effective amount of the antibody of claim 27.

31. A method for modulating the endogenous activity of an EPO receptor in a mammal comprising administering a modulating effective amount of the antibody of claim 27.

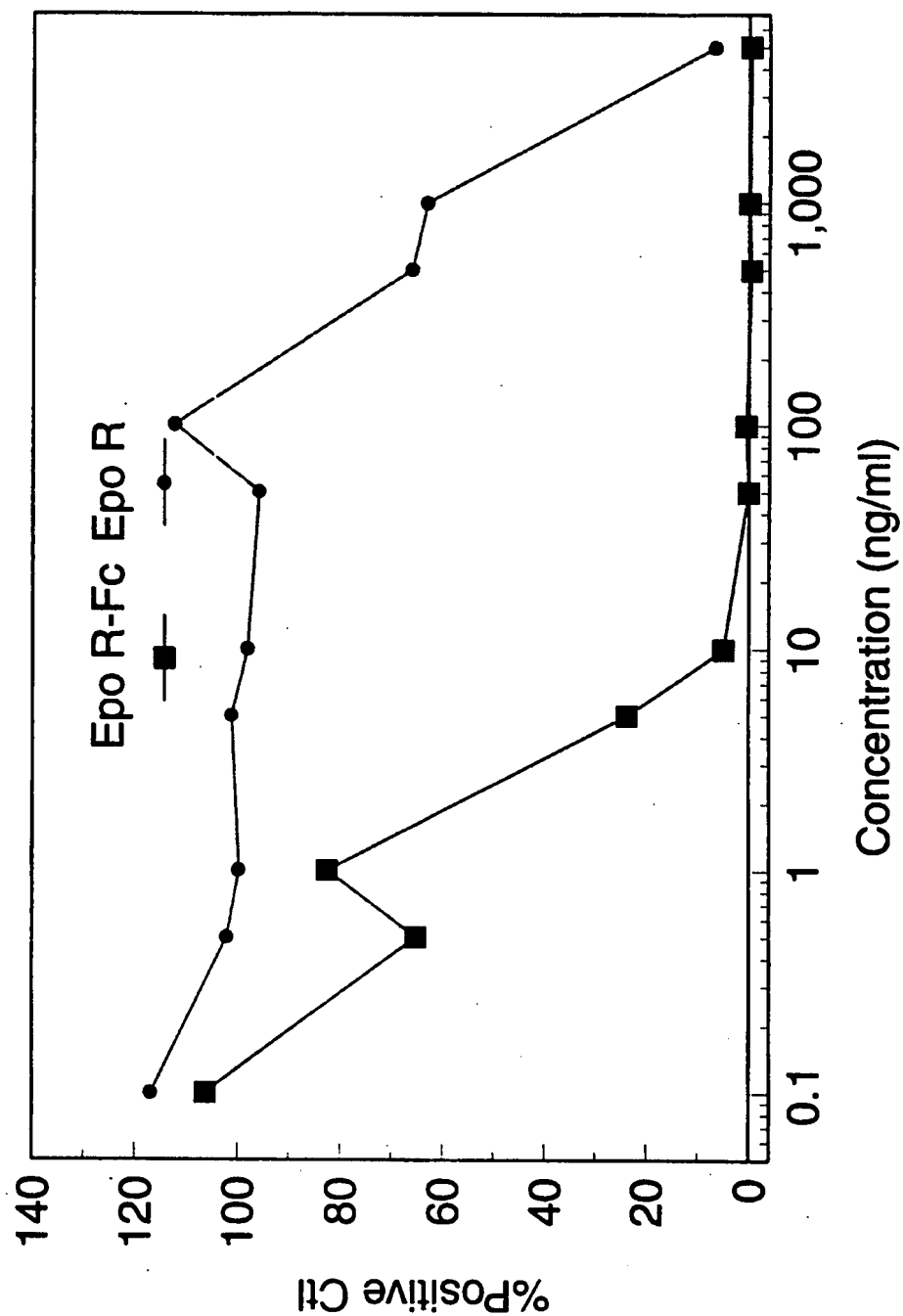


Fig. 1

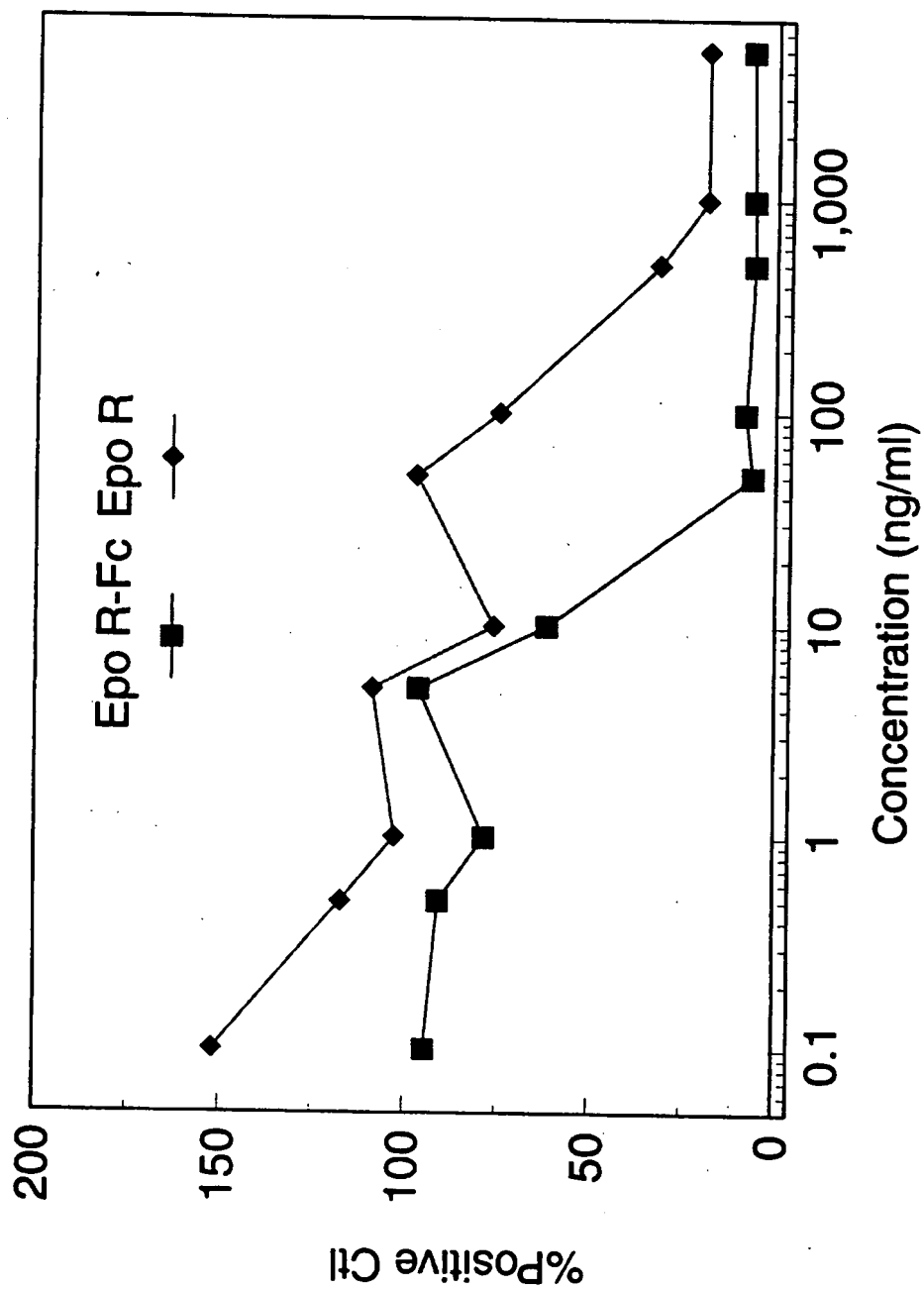


Fig. 2



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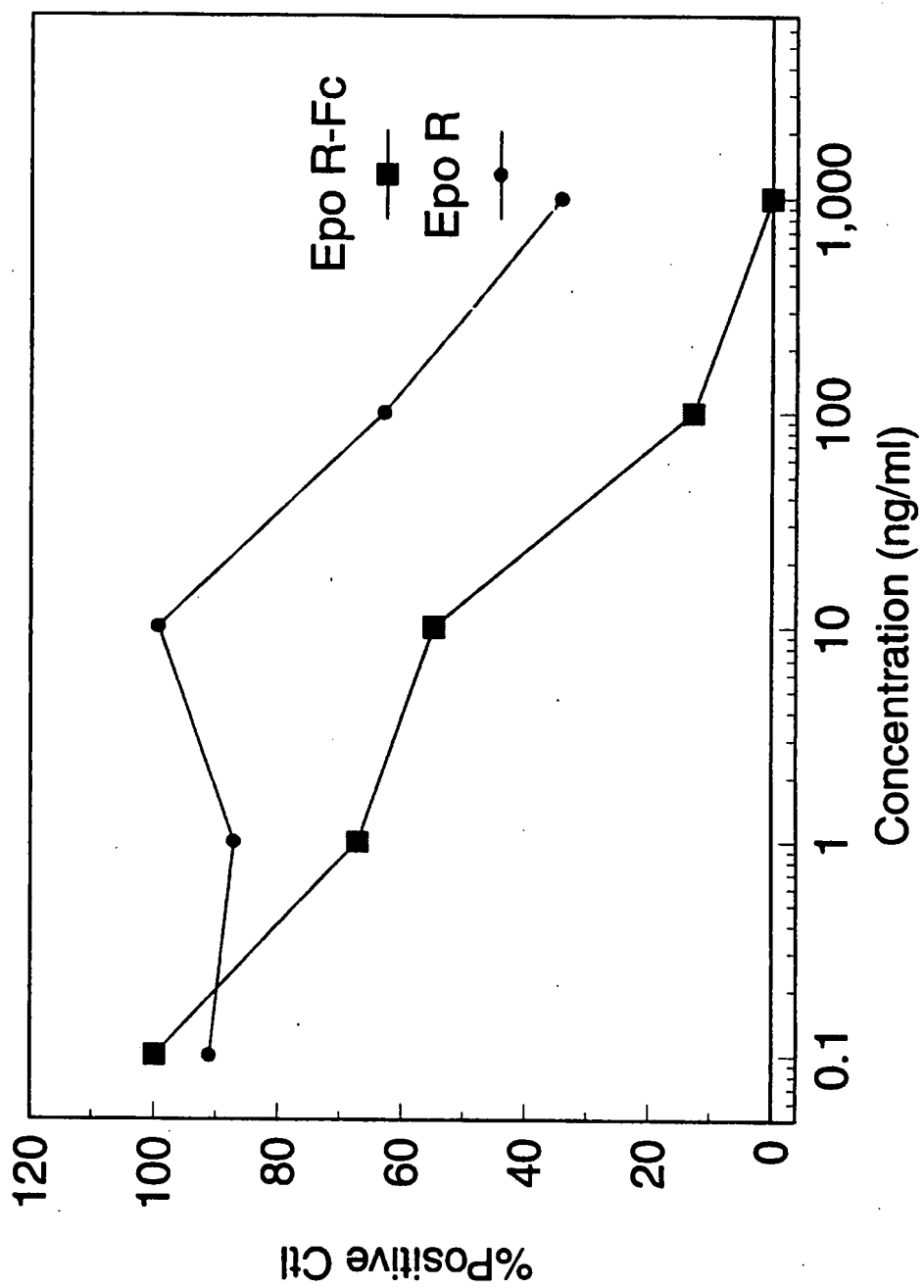
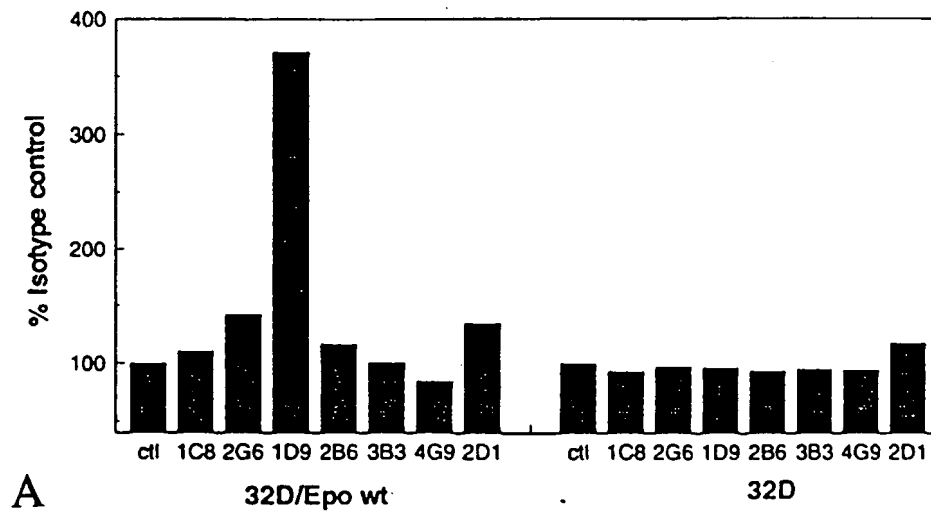


Fig. 3

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### Epo R-Fc McAb Binding to 32D/epo wt and Parental Cells



### Epo R-Fc McAb Binding to UT7-Epo Cells

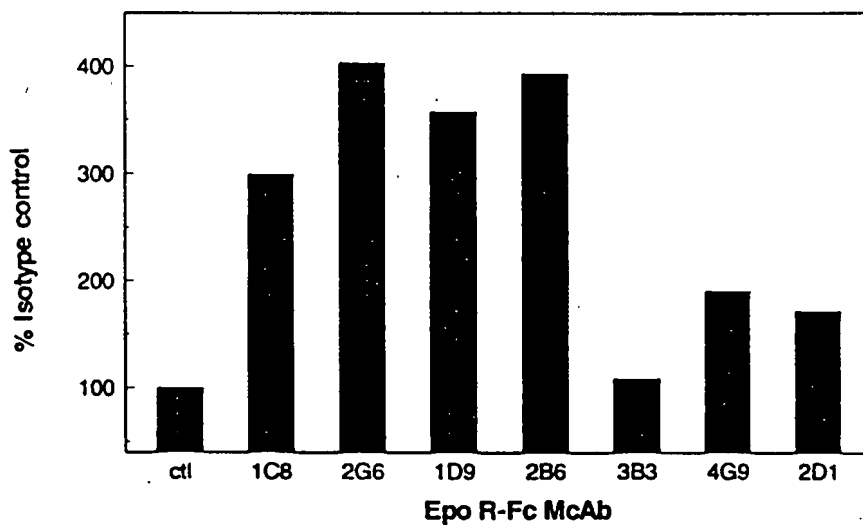


Fig. 4

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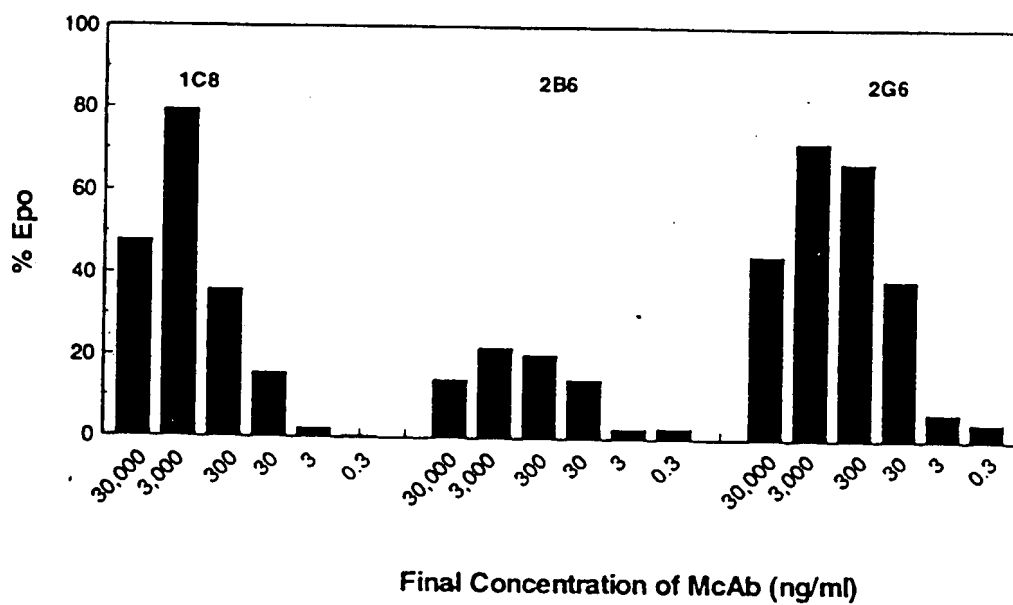


Fig. 5

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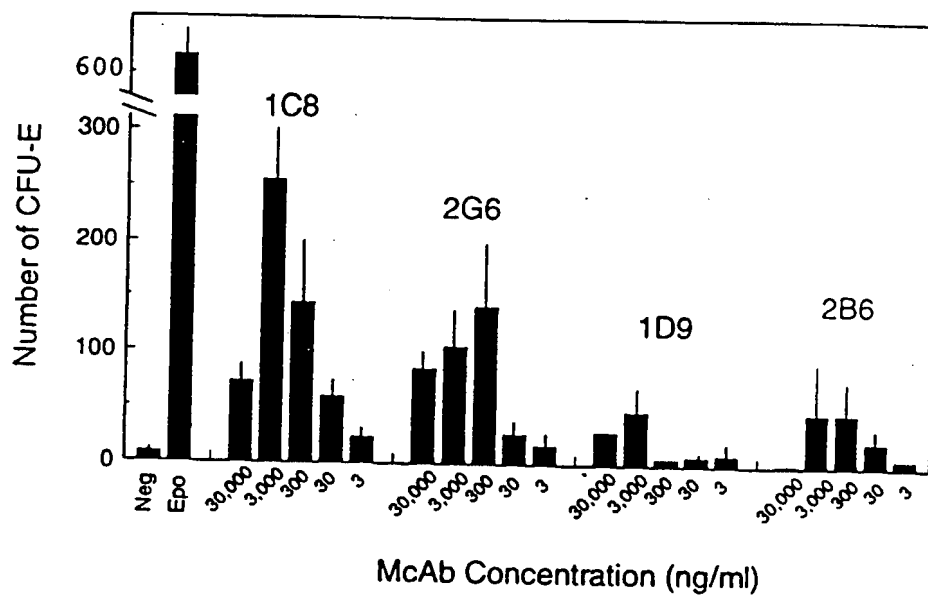


Fig. 6

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plasmid mtalsEpoRFc [SEQ ID NO: 1 and 2]

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GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCC	100
TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG	150
CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA	200
CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCCGCATT	250
CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT	300
TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA	350
ACGCCAGGGT TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGCCAG	400
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CCCCACCGCC CACCGCCACC CCCATACATA TGTGGTACGC AAGTAAGAGT	550
GCCTGCGCAT GCCCCATGTG CCCCACCAAG AGTTTTGCAT CCCATACAAG	600
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CTGCACACGT CTCCACTCGA ATTTGGAGCC GGCCGGCGTG TGCAAAAGAG	700
GTGAATCGAA CGAAAGACCC GTGTGTAAAG CCGCGTTTCC AAAATGTATA	750
AAACCGAGAG CATCTGGCCA ATGTGCATCA GTTGTGGTCA GCAGCAAAAT	800
CAAGTGAATC ATCTCAGTGC AACTAAAGGG GGGATCCGAT ATCCAAGGTT	850
ACCGCGGACT AGTCTAGTAA CGGCCGCCAG TGTGCTGGAA TTCGGCT ATG	900
	Met 1
GAC CAC CTC GGG GCG TCC CTC TGG CCC CAG GTC GGC TCC CTT	942
Asp His Leu Gly Ala Ser Leu Trp Pro Gln Val Gly Ser Leu	5 10 15
TGT CTC CTG CTC GCT GGG GCC GCC TGG GCG CCC CCG CCT AAC	984
Cys Leu Leu Leu Ala Gly Ala Ala Trp Ala Pro Pro Pro Asn	20 25
CTC CCG GAC CCC AAG TTC GAG AGC AAA GCG GCC TTG CTG GCG	1026
Leu Pro Asp Pro Lys Phe Glu Ser Lys Ala Ala Leu Leu Ala	30 35 40

Fig. 7A

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GCC	CGG	GGG	CCC	GAA	GAG	CTT	CTG	TGC	TTC	ACC	GAG	CGG	TTG	1068
Ala	Arg	Gly	Pro	Glu	Glu	Leu	Leu	Cys	Phe	Thr	Glu	Arg	Leu	
	45					50					55			
GAG	GAC	TTG	GTG	TGT	TTC	TGG	GAG	GAA	GCG	GCG	AGC	GCT	GGG	1110
Glu	Asp	Leu	Val	Cys	Phe	Trp	Glu	Glu	Ala	Ala	Ser	Ala	Gly	
		60					65					70		
GTG	GGC	CCG	GGC	AAC	TAC	AGC	TTC	TCC	TAC	CAG	CTC	GAG	GAT	1152
Val	Gly	Pro	Gly	Asn	Tyr	Ser	Phe	Ser	Tyr	Gln	Leu	Glu	Asp	
			75					80					85	
GAG	CCA	TGG	AAG	CTG	TGT	CGC	CTG	CAC	CAG	GCT	CCC	ACG	GCT	1194
Glu	Pro	Trp	Lys	Leu	Cys	Arg	Leu	His	Gln	Ala	Pro	Thr	Ala	
				90					95					
CGT	GGT	GCG	GTG	CGC	TTC	TGG	TGT	TCG	CTG	CCT	ACA	GCC	GAC	1236
Arg	Gly	Ala	Val	Arg	Phe	Trp	Cys	Ser	Leu	Pro	Thr	Ala	Asp	
100					105					110				
ACG	TCG	AGC	TTC	GTG	CCC	CTA	GAG	TTG	CGC	GTC	ACA	GCA	GCC	1278
Thr	Ser	Ser	Phe	Val	Pro	Leu	Glu	Leu	Arg	Val	Thr	Ala	Ala	
	115					120					125			
TCC	GGC	GCT	CCG	CGA	TAT	CAC	CGT	GTC	ATC	CAC	ATC	AAT	GAA	1320
Ser	Gly	Ala	Pro	Arg	Tyr	His	Arg	Val	Ile	His	Ile	Asn	Glu	
		130					135					140		
GTA	GTG	CTC	CTA	GAC	GCC	CCC	GTG	GGG	CTG	GTG	GCG	CGG	TTG	1362
Val	Val	Leu	Leu	Asp	Ala	Pro	Val	Gly	Leu	Val	Ala	Arg	Leu	
			145					150					155	
GCT	GAC	GAG	AGC	GGC	CAC	GTA	GTG	TTG	CGC	TGG	CTC	CCG	CCG	1404
Ala	Asp	Glu	Ser	Gly	His	Val	Val	Leu	Arg	Trp	Leu	Pro	Pro	
				160					165					
CCT	GAG	ACA	CCC	ATG	ACG	TCT	CAC	ATC	CGC	TAC	GAG	GTG	GAC	1446
Pro	Glu	Thr	Pro	Met	Thr	Ser	His	Ile	Arg	Tyr	Glu	Val	Asp	
170					175					180				
GTC	TCG	GCC	GGC	AAC	GGC	GCA	GGG	AGC	GTA	CAG	AGG	GTG	GAG	1488
Val	Ser	Ala	Gly	Asn	Gly	Ala	Gly	Ser	Val	Gln	Arg	Val	Glu	
	185					190					195			
ATC	CTG	GAG	GGC	CGC	ACC	GAG	TGT	GTG	CTG	AGC	AAC	CTG	CGG	1530
Ile	Leu	Glu	Gly	Arg	Thr	Glu	Cys	Val	Leu	Ser	Asn	Leu	Arg	
		200					205					210		
GGC	CGG	ACG	CGC	TAC	ACC	TTC	GCC	GTC	CGC	GCG	CGT	ATG	GCT	1572
Gly	Arg	Thr	Arg	Tyr	Thr	Phe	Ala	Val	Arg	Ala	Arg	Met	Ala	
			215					220					225	

Fig. 7B

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GAG	CCG	AGC	TTC	GGC	GGC	TTC	TGG	AGC	GCC	TGG	TCG	GAG	CCT	1614
Glu	Pro	Ser	Phe	Gly	Gly	Phe	Trp	Ser	Ala	Trp	Ser	Glu	Pro	
				230					235					
GTG	TCG	CTG	CTG	ACG	CCT	AGC	GAC	CTG	GAC	CCC	ATT	GAG	GGC	1656
Val	Ser	Leu	Leu	Thr	Pro	Ser	Asp	Leu	Asp	Pro	Ile	Glu	Gly	
240					245					250				
CGT	GGT	ACC	GAG	CCC	AAA	TCG	GCC	GAC	AAA	ACT	CAC	ACA	TGC	1698
Arg	Gly	Thr	Glu	Pro	Lys	Ser	Ala	Asp	Lys	Thr	His	Thr	Cys	
	255					260					265			
CCA	CCG	TGC	CCA	GCA	CCT	GAA	CTC	CTG	GGG	GGA	CCG	TCA	GTC	1740
Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	
		270					275					280		
TTC	CTC	TTC	CCC	CCA	AAA	CCC	AAG	GAC	ACC	CTC	ATG	ATC	TCC	1782
Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	
			285					290					295	
CGG	ACC	CCT	GAG	GTC	ACA	TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	1824
Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	
				300					305					
GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC	GGC	GTG	1866
Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	
310					315					320				
GAG	GTG	CAT	AAT	GCC	AAG	ACA	AAG	CCG	CGG	GAG	GAG	CAG	TAC	1908
Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	
	325					330					335			
AAC	AGC	ACG	TAC	CGG	GTG	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	1950
Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	
		340					345					350		
CAG	GAC	TGG	CTG	AAT	GGC	AAG	GAG	TAC	AAG	TGC	AAG	GTC	TCC	1992
Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	
			355					360					365	
AAC	AAA	GCC	CTC	CCA	GCC	CCC	ATC	GAG	AAA	ACC	ATC	TCC	AAA	2034
Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	
				370					375					
GCC	AAA	GGG	CAG	CCC	CGA	GAA	CCA	CAG	GTG	TAC	ACC	CTG	CCC	2076
Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	
380					385					390				
CCA	TCC	CGG	GAT	GAG	CTG	ACC	AAG	AAC	CAG	GTC	AGC	CTG	ACC	2118
Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	
	395					400					405			

Fig. 7C

10/17

TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG	2160
Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu	
410 415 420	
TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG	2202
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr	
425 430 435	
CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC	2244
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser	
440 445	
AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC	2286
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val	
450 455 460	
TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC	2328
Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr	
465 470 475	
ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGAGTGTAGT	2371
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys	
480 485	
CTAGAAGCTT ACGCGTAGGC CTGAGCTCGC TGATCAGCCT CGAGGATCCA	2421
GACATGATAA GATACATTGA TGAGTTTGGA CAAACCACAA CTAGAATGCA	2471
GTGAAAAAAA TGCTTTATTT GTGAAATTTG TGATGCTATT GCTTTATTTG	2521
TAACCATTAT AAGCTGCAAT AAACAAGTTA ACAACAACAA TTGCATT CAT	2571
TTTATGTTTC AGGTTCAGGG GGAGGTGTGG GAGGTTTTTTT AAAGCAAGTA	2621
AAACCTCTAC AAATGTGGTA TGGCTGATTA TGATCAGTCG ACCGATGCCC	2671
TTGAGAGCCT TCAACCCAGT CAGCTCCTTC CGGTGGGCGC GGGGCATGAC	2721
TATCGTCGCC GCACTTATGA CTGTCTTCTT TATCATGCAA CTCGTAGGAC	2771
AGGTGCCCGC AGCGCTCTGG GTCATTTTCG GCGAGGACCG CTTTCGCTGG	2821
AGCGCGACGA TGATCGGCCT GTCGCTTGCG GTATTCGGAA TCTTGACGC	2871
CCTCGCTCAA GCCTTCGTCA CTGGTCCCGC CACCAAACGT TTCGGCGAGA	2921
AGCAGGCCAT TATCGCCGGC ATGGCGGCCG ACGCGCTGGG CTACGTCTTG	2971
CTGGCGTTTCG CGACGCGAGG CTGGATGGCC TTCCCCATTA TGATTCTTCT	3021
CGCTTCCGGC GGCATCGGGA TGCCCGCGTT GCAGGCCATG CTGTCCAGGC	3071

Fig. 7D

**SUBSTITUTE SHEET (RULE 26)**



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AGGTAGATGA	CGACCATCAG	GGACAGCTTC	AAGGATCGCT	CGCGGCTCTT	3121
ACCAGCCAGC	AAAAGGCCAG	GAACCGTAAA	AAGGCCGCGT	TGCTGGCGTT	3171
TTTCCATAGG	CTCCGCCCCC	CTGACGAGCA	TCACAAAAAT	CGACGCTCAA	3221
GTCAGAGGTG	GCGAAACCCG	ACAGGACTAT	AAAGATACCA	GGCGTTTCCC	3271
CCTGGAAGCT	CCCTCGTGCG	CTCTCCTGTT	CCGACCCTGC	CGCTTACCGG	3321
ATACCTGTCC	GCCTTTCTCC	CTTCGGGAAG	CGTGGCGCTT	TCTCAATGCT	3371
CACGCTGTAG	GTATCTCAGT	TCGGTGTAGG	TCGTTGCTC	CAAGCTGGGC	3421
TGTGTGCACG	AACCCCCCGT	TCAGCCCGAC	CGCTGCGCCT	TATCCGGTAA	3471
CTATCGTCTT	GAGTCCAACC	CGGTAAGACA	CGACTTATCG	CCACTGGCAG	3521
CAGCCACTGG	TAACAGGATT	AGCAGAGCGA	GGTATGTAGG	CGGTGCTACA	3571
GAGTTCTTGA	AGTGGTGGCC	TAACACGGC	TACACTAGAA	GGACAGTATT	3621
TGGTATCTGC	GCTCTGCTGA	AGCCAGTTAC	CTTCGGAAAA	AGAGTTGGTA	3671
GCTCTTGATC	CGGCAAACAA	ACCACCGCTG	GTAGCGGTGG	TTTTTTTGTT	3721
TGCAAGCAGC	AGATTACGCG	CAGAAAAAAA	GGATCTCAAG	AAGATCCTTT	3771
GATCTTTTCT	ACGGGGTCTG	ACGCTCAGTG	GAACGAAAAC	TCACGTAAAG	3821
GGATTTTGGT	CATGAGATTA	TCAAAAAGGA	TCTTCACCTA	GATCCTTTTA	3871
AATTAAAAAT	GAAGTTTTAA	ATCAATCTAA	AGTATATATG	AGTAAACTTG	3921
GTCTGACAGT	TACCAATGCT	TAATCAGTGA	GGCACCTATC	TCAGCGATCT	3971
GTCTATTTCTG	TTCATCCATA	GTTGCCTGAC	TCCCCGTCGT	GTAGATAACT	4021
ACGATACGGG	AGGGCTTACC	ATCTGGCCCC	AGTGCTGCAA	TGATACCGCG	4071
AGACCCACGC	TCACCGGCTC	CAGATTTATC	AGCAATAAAC	CAGCCAGCCG	4121
GAAGGGCCGA	GCGCAGAAGT	GGTCCTGCAA	CTTTATCCGC	CTCCATCCAG	4171
TCTATTAAAT	GTTGCCGGGA	AGCTAGAGTA	AGTAGTTCGC	CAGTTAATAG	4221
TTTGCGCAAC	GTTGTTGCCA	TTGCTGCAGG	CATCGTGGTG	TCACGCTCGT	4271
CGTTTGGTAT	GGCTTCATTC	AGCTCCGGTT	CCCAACGATC	AAGGCGAGTT	4321
ACATGATCCC	CCATGTTGTG	CAAAAAGCG	GTTAGCTCCT	TCGGTCCTCC	4371

Fig. 7E

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GATCGTTGTC	AGAAGTAAGT	TGGCCGCAGT	GTTATCACTC	ATGGTTATGG	4421
CAGCACTGCA	TAATTCTCTT	ACTGTCATGC	CATCCGTAAG	ATGCTTTTCT	4471
GTGACTGGTG	AGTACTCAAC	CAAGTCATTC	TGAGAATAGT	GTATGCGGCG	4521
ACCGAGTTGC	TCTTGCCCGG	CGTCAACACG	GGATAATACC	GCGCCACATA	4571
GCAGAACTTT	AAAAGTGCTC	ATCATTGGAA	AACGTTCTTC	GGGGCGAAAA	4621
CTCTCAAGGA	TCTTACCGCT	GTTGAGATCC	AGTTCGATGT	AACCCACTCG	4671
TGCACCCAAC	TGATCTTCAG	CATCTTTTAC	TTTCACCAGC	GTTTCTGGGT	4721
GAGCAAAAAC	AGGAAGGCAA	AATGCCGCAA	AAAAGGGAAT	AAGGGCGACA	4771
CGGAAATGTT	GAATACTCAT	ACTCTTCCTT	TTTCAATATT	ATTGAAGCAT	4821
TTATCAGGGT	TATTGTCTCA	TGAGCGGATA	CATATTTGAA	TGTATTTAGA	4871
AAAATAAACA	AATAGGGGTT	CCGCGCACAT	TTCCCCGAAA	AGTGCCACCT	4921
GACGTCTAAG	AAACCATTAT	TATCATGACA	TTAACCTATA	AAAATAGGCG	4971
TATCACGAGG	CCCTTTCGT				4990

Fig. 7F

**SUBSTITUTE SHEET (RULE 26)**

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COSFclink [SEQ ID NO: 3]

GACGTCGACG	GATCGGGAGA	TCGGGGATCG	ATCCGTCGAC	GTACGACTAG	50
TTATTAATAG	TAATCAATTA	CGGGGTCATT	AGTTCATAGC	CCATATATGG	100
AGTTCCGCGT	TACATAACTT	ACGGTAAATG	GCCCCGCTGG	CTGACCGCCC	150
AACGACCCCC	GCCCATTGAC	GTCAATAATG	ACGTATGTTC	CCATAGTAAC	200
GCCAATAGGG	ACTTTCCATT	GACGTCAATG	GGTGGACTAT	TTACGGTAAA	250
CTGCCCCTT	GGCAGTACAT	CAAGTGTATC	ATATGCCAAG	TACGCCCCCT	300
ATTGACGTCA	ATGACGGTAA	ATGGCCCGCC	TGGCATTATG	CCCAGTACAT	350
GACCTTATGG	GACTTTCCTA	CTTGGCAGTA	CATCTACGTA	TTAGTCATCG	400
CTATTACCAT	GGTGATGCGG	TTTTGGCAGT	ACATCAATGG	GCGTGGATAG	450
CGGTTTGA	CT	CAAGTCTC	CACCCCAT	ACGTCAATGG	500
GAGTTTGTTT	TGGCACCAA	ATCAACGGGA	CTTTCCAAA	TGTCGTAACA	550
ACTCCGCCCC	ATTGACGCAA	ATGGGCGGTA	GGCGTGACG	GTGGGAGGTC	600
TATATAAGCA	GAGCTGGGTA	CGTGAACCGT	CAGATCGCCT	GGAGACGCCA	650
TCGAATTCGG	TTACCTGCAG	ATATCAAGCT	AATTCGGTAC	CGAGCCCAA	700
TCGGCCGACA	AAACTCACAC	ATGCCACCG	TGCCCAGCAC	CTGAACTCCT	750
GGGGGGACCG	TCAGTCTTCC	TCTTCCCCC	AAAACCCAAG	GACACCCTCA	800
TGATCTCCCG	GACCCCTGAG	GTCACATGCG	TGGTGGTGGA	CGTGAGCCAC	850
GAAGACCCTG	AGGTCAAGTT	CAACTGGTAC	GTGGACGGCG	TGGAGGTGCA	900
TAATGCCAAG	ACAAAGCCGC	GGGAGGAGCA	GTACAACAGC	ACGTACCGGG	950
TGGTCAGCGT	CCTCACCGTC	CTGCACCAGG	ACTGGCTGAA	TGGCAAGGAG	1000
TACAAGTGCA	AGGTCTCCAA	CAAAGCCCTC	CCAGCCCCCA	TCGAGAAAAC	1050
CATCTCCAAA	GCCAAAGGGC	AGCCCCGAGA	ACCACAGGTG	TACACCCTGC	1100
CCCCATCCCG	GGATGAGCTG	ACCAAGAACC	AGGTCAGCCT	GACCTGCCTG	1150
GTCAAAGGCT	TCTATCCCAG	CGACATCGCC	GTGGAGTGGG	AGAGCAATGG	1200
GCAGCCGGAG	AACAACCTACA	AGACCACGCC	TCCCGTGCTG	GACTCCGACG	1250

Fig. 8A

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GCTCCTTCTT	CCTCTACAGC	AAGCTCACCG	TGGACAAGAG	CAGGTGGCAG	1300
CAGGGGAACG	TCTTCTCATG	CTCCGTGATG	CATGAGGCTC	TGCACAACCA	1350
CTACACGCAG	AAGAGCCTCT	CCCTGTCTCC	GGGTAAATGA	GTGTAGTCTA	1400
GAGCTCGCTG	ATCAGCCTCG	ACTGTGCCTT	CTAGTTGCCA	GCCATCTGTT	1450
GTTTGCCCCCT	CCCCCGTGCC	TTCCTTGACC	CTGGAAGGTG	CCACTCCCAC	1500
TGTCCTTTCC	TAATAAAATG	AGGAAATTGC	ATCGCATTGT	CTGAGTAGGT	1550
GTCATTCTAT	TCTGGGGGGT	GGGGTGGGGC	AGGACAGCAA	GGGGGAGGAT	1600
TGGGAAGACA	ATAGCAGGCA	TGCTGGGGAT	GCGGTGGGCT	CTATGGAACC	1650
AGCTGGGGCT	CGAGGGGGGA	TCTCCCGATC	CCCAGCTTTG	CTTCTCAATT	1700
TCTTATTTGC	ATAATGAGAA	AAAAAGGAAA	ATTAATTTTA	ACACCAATTC	1750
AGTAGTTGAT	TGAGCAAATG	CGTTGCCAAA	AAGGATGCTT	TAGAGACAGT	1800
GTTCTCTGCA	CAGATAAGGA	CAAACATTAT	TCAGAGGGAG	TACCCAGAGC	1850
TGAGACTCCT	AAGCCAGTGA	GTGGCACAGC	ATTCTAGGGA	GAAATATGCT	1900
TGTCATCACC	GAAGCCTGAT	TCCGTAGAGC	CACACCTTGG	TAAGGGCCAA	1950
TCTGCTCACA	CAGGATAGAG	AGGGCAGGAG	CCAGGGCAGA	GCATATAAGG	2000
TGAGGTAGGA	TCAGTTGCTC	CTCACATTTG	CTTCTGACAT	AGTTGTGTTG	2050
GGAGCTTGGA	TAGCTTGGAC	AGCTCAGGGC	TGCGATTTCG	CGCCAAACTT	2100
GACGGCAATC	CTAGCGTGAA	GGCTGGTAGG	ATTTTATCCC	CGCTGCCATC	2150
ATGGTTCGAC	CATTGAACTG	CATCGTCGCC	GTGTCCCAAA	ATATGGGGAT	2200
TGGCAAGAAC	GGAGACCTAC	CCTGGCCTCC	GCTCAGGAAC	GAGTTCAAGT	2250
ACTTCCAAAG	AATGACCACA	ACCTCTTCAG	TGGAAGGTAA	ACAGAATCTG	2300
GTGATTATGG	GTAGGAAAAC	CTGGTTCTCC	ATTCCTGAGA	AGAATCGACC	2350
TTTAAAGGAC	AGAATTAATA	TAGTTCTCAG	TAGAGAACTC	AAAGAACCAC	2400
CACGAGGAGC	TCATTTTCTT	GCCAAAAGTT	TGGATGATGC	CTTAAGACTT	2450
ATTGAACAAC	CGGAATTGGC	AAGTAAAGTA	GACATGGTTT	GGATAGTCGG	2500
AGGCAGTTCT	GTTTACCAGG	AAGCCATGAA	TCAACCAGGC	CACCTTAGAC	2550

Fig. 8B

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TCTTTGTGAC	AAGGATCATG	CAGGAATTTG	AAAGTGACAC	GTTTTTCCCA	2600
GAAATTGATT	TGGGGAAATA	TAAACTTCTC	CCAGAATACC	CAGGCGTCCT	2650
CTCTGAGGTC	CAGGAGGAAA	AAGGCATCAA	GTATAAGTTT	GAAGTCTACG	2700
AGAAGAAAGA	CTAACAGGAA	GATGCTTTCA	AGTTCTCTGC	TCCCCTCCTA	2750
AAGCTATGCA	TTTTTATAAG	ACCATGCTAG	CTTGAAGTTG	TTTATTGCAG	2800
CTTATAATGG	TTACAAATAA	AGCAATAGCA	TCACAAATTT	CACAAATAAA	2850
GCATTTTTTTT	CACTGCATTC	TAGTTGTGGT	TTGTCCAAAC	TCATCAATGT	2900
ATCTTATCAT	GTCTGGATCA	ACGATAGCTT	ATCTGTGGGC	GATGCCAAGC	2950
ACCTGGATGC	TGTTGGTTTC	CTGCTACTGA	TTAGAAGCC	ATTTGCCCCC	3000
TGAGTGGGGC	TTGGGAGCAC	TAACTTTCTC	TTTCAAAGGA	AGCAATGCAG	3050
AAAGAAAAGC	ATACAAAGTA	TAAGCTGCCA	TGTAATAATG	GAAGAAGATA	3100
AGGTTGTATG	AATTAGATTT	ACATACTTCT	GAATTGAAAC	TAAACACCTT	3150
TAAATTCTTA	AATATATAAC	ACATTTTATA	TGAAAGTATT	TTACATAAGT	3200
AACTCAGATA	CATAGAAAAC	AAAGCTAATG	ATAGGTGTCC	CTAAAAGTTC	3250
ATTTATTAAT	TCTACAAATG	ATGAGCTGGC	CATCAAAATT	CCAGCTCAAT	3300
TCTTCAACGA	ATTAGAAAGA	GCAATCTGCA	AACTCATCTG	GAATAACAAA	3350
AAACCTAGGA	TAGCAAAAAC	TCTTCTCAAG	GATAAAAGAA	CCTCTGGTGG	3400
AATCACCATG	CCTGACCTAA	AGCTGTACTA	CAGAGCAATT	GTGATAAAAA	3450
CTGCATGGTA	CTGATATAGA	AACGGACAAG	TAGACCAATG	GAATAGAACC	3500
CACACACCTA	TGGTCACTTG	ATCTTCAACA	AGAGAGCTAA	AACCATCCAC	3550
TGGAAAAAAG	ACAGCATTTT	CAACAAATGG	TGCTGGCACA	ACTGGTGGTT	3600
ATCATGGAGA	AGAATGTGAA	TTGATCCATT	CCAATCTCCT	TGTACTAAGG	3650
TCAAATCTAA	GTGGATCAAG	GAAGTCCACA	TAAAACCAGA	GAACTGAAA	3700
CTTATAGAGG	AGAAAGTGGG	GAAAAGCCTC	GAAGATATGG	GCACAGGGGA	3750
AAAATTCCTG	AATAGAACAG	CAATGGCTTG	TGCTGTAAGA	TCGAGAATTG	3800
ACAAATGGGA	CCTCATGAAA	CTCCAAAGCT	ATCGGATCAA	TTCCTCCAAA	3850

Fig. 8C

SUBSTITUTE SHEET (RULE 26)

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AAAGCCTCCT	CACTACTTCT	GGAATAGCTC	AGAGGCCGAG	GCGGCCTCGG	3900
CCTCTGCATA	AATAAAAAAA	ATTAGTCAGC	CATGCATGGG	GCGGAGAATG	3950
GGCGGAACTG	GGCGGAGTTA	GGGGCGGGAT	GGGCGGAGTT	AGGGGCGGGA	4000
CTATGGTTGC	TGACTAATTG	AGATGCATGC	TTTGCATACT	TCTGCCTGCT	4050
GGGGAGCCTG	GGGACTTTCC	ACACCTGGTT	GCTGACTAAT	TGAGATGCAT	4100
GCTTTGCATA	CTTCTGCCTG	CTGGGGAGCC	TGGGGACTTT	CCACACCCTA	4150
ACTGACACAC	ATTCCACAGA	ATTAATTCCC	GATCCCGTCG	ACCTCGAGAG	4200
CTTGGCGTAA	TCATGGTCAT	AGCTGTTTCC	TGTGTGAAAT	TGTTATCCGC	4250
TCACAATTCC	ACACAACATA	CGAGCCGGAA	GCATAAAGTG	TAAAGCCTGG	4300
GGTGCCTAAT	GAGTGAGCTA	ACTCACATTA	ATTGCGTTGC	GCTCACTGCC	4350
CGCTTTCCAG	TCGGGAAACC	TGTCGTGCCA	GCTGCATTAA	TGAATCGGCC	4400
AACGCGCGGG	GAGAGGCGGT	TTGCGTATTG	GGCGCTCTTC	CGCTTCCTCG	4450
CTCACTGACT	CGCTGCGCTC	GGTCGTTCCG	CTGCGGCGAG	CGGTATCAGC	4500
TCACTCAAAG	GCGGTAATAC	GGTTATCCAC	AGAATCAGGG	GATAACGCAG	4550
GAAAGAACAT	GTGAGCAAAA	GGCCAGCAAA	AGGCCAGGAA	CCGTAAAAAG	4600
GCCGCGTTGC	TGGCGTTTTT	CCATAGGCTC	CGCCCCCTG	ACGAGCATCA	4650
CAAAAATCGA	CGCTCAAGTC	AGAGGTGGCG	AAACCCGACA	GGACTATAAA	4700
GATACCAGGC	GTTTCCCCCT	GGAAGCTCCC	TCGTGCGCTC	TCCTGTTCCG	4750
ACCCTGCCGC	TTACCGGATA	CCTGTCCGCC	TTTCTCCCTT	CGGGAAGCGT	4800
GGCGCTTTCT	CAATGCTCAC	GCTGTAGGTA	TCTCAGTTTC	GTGTAGGTCG	4850
TTCGCTCCAA	GCTGGGCTGT	GTGCACGAAC	CCCCCGTTCA	GCCCGACCGC	4900
TGCGCCTTAT	CCGGTAACTA	TCGTCTTGAG	TCCAACCCGG	TAAGACACGA	4950
CTTATCGCCA	CTGGCAGCAG	CCACTGGTAA	CAGGATTAGC	AGAGCGAGGT	5000
ATGTAGGCGG	TGCTACAGAG	TTCTTGAAGT	GGTGGCCTAA	CTACGGCTAC	5050
ACTAGAAGGA	CAGTATTTGG	TATCTGCGCT	CTGCTGAAGC	CAGTTACCTT	5100

Fig. 8D

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CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA	5150
GCGGTGGTTT TTTTGTTCG AAGCAGCAGA TTACGCGCAG AAAAAAGGA	5200
TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA	5250
CGAAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA AAAAGGATCT	5300
TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT	5350
ATATATGAGT AACTTTGGTC TGACAGTTAC CAATGCTTAA TCAGTGAGGC	5400
ACCTATCTCA GCGATCTGTC TATTTTCGTT ATCCATAGTT GCCTGACTCC	5450
CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC TGGCCCCAGT	5500
GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC	5550
AATAAACCCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT	5600
TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGAAGC TAGAGTAAGT	5650
AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG CTACAGGCAT	5700
CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTACAGC TCCGGTCCC	5750
AACGATCAAG GCGAGTTACA TGATCCCCCA TGTTGTGCAA AAAAGCGGTT	5800
AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGT	5850
ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT	5900
CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA	5950
GAATAGTGTA TGCGGCGACC GAGTTGCTCT TGCCCCGCGT CAATACGGGA	6000
TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC	6050
GTTCTTCGGG GCGAAAACTC TCAAGGATCT TACCGCTGTT GAGATCCAGT	6100
TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTTACTTT	6150
CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT GCCGAAAAA	6200
AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT	6250
CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA GCGGATACAT	6300
ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCGG CGCACATTTT	6350
CCCGAAAAGT GCCACCT	6367

Fig. 8E

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09613

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/133.1, 192.1, 194.1; 530/387.1; 435/240.27, 172.3, 70.21, 71.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, BIOSIS, SCISEARCH, EMBASE, MEDLINE, WPI, search terms: immunogen, antigen, extracellular, surface, receptor, antibody, immunoglobulin, erythropoietin, dimer, authors.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Immunol., Volume 142, Number 3, issued 01 February 1989, SCHREURS et al., "A monoclonal antibody with IL-3-like activity blocks IL-3 binding and stimulates tyrosine phosphorylation", pages 819-825, see entire document.	1-31
Y	Blood, Volume 82, Number 6, issued 15 September 1993, YET et al., "The extracytoplasmic domain of the erythropoietin receptor forms a monomeric complex with erythropoietin", pages 1713-1719, see entire document.	1-31



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 AUGUST 1996

Date of mailing of the international search report

13 SEP 1996

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09613

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 256, issued 19 June 1992, FUH et al., "Rational design of potent antagonists to the human growth hormone receptor", pages 1677-1680, see entire document.	1-31
Y	Proc. Natl. Acad. Sci. USA, Volume 89, issued March 1992, WATOWICH et al., "Homodimerization and constitutive activation of the erythropoietin receptor", pages 2140-2144, see entire document.	1-31
Y	J. Immunol., Volume 140, Number 2, issued January 1992, SUGAWARA et al., "Monoclonal autoantibodies with interleukin 3-like activity derived from a MRL/lpr mouse", pages 526-530, see entire document.	1-31
Y	Blood, Volume 82, Number 1, issued 01 July 1993, D'ANDREA et al., "Anti-erythropoietin receptor (EPO-R) antibodies inhibit erythropoietin binding and neutralize bioactivity", pages 46-52, see entire document.	1-31

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09613

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09613

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 39/00, 39/385, 39/395, 39/40, 39/42; C07K 16/00; C12N 5/00, 15/00; C12P 21/04

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/133.1, 192.1, 194.1; 530/387.1; 435/240.27, 172.3, 70.21, 71.1

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Species 1. Immunogens having a bridging moiety which is peptidic; and

Species 2. Immunogens having a bridging moiety which is an organic non-peptidic molecule.

The claims are deemed to correspond to the species listed above in the following manner:

Species 1, claims 4-7, 17 and 19-31, and

Species 2, claims 8-10 and 18

The following claims are generic: 1-3 and 11-16.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: immunogens having bridges such as peptides are structurally different from those immunogens having organic non-peptidic linkers. Further, the immunogens are made differently, using materially different process steps and reagents.